

Bacterial electrical routes: deciphering extracellular electron transfer pathways in *Geobacter*

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ABSTRACT

Geobacter bacteria exhibit remarkable respiratory versatility, sustaining their growth through several extracellular compounds in contrast to conventional respiratory processes involving soluble electron donors and acceptors, which is reflected in a significantly impact on biotechnological applications such as bioremediation of organic and inorganic contaminants and bioenergy production. To optimize these applications, it is imperative to understand the mechanism of electron exchange between the cell and the external environment, a process known as extracellular electron transfer (EET). Periplasmic *c*-type triheme cytochromes have been identified as essential components for EET by bridging the inner and outer membrane proteins.

This Thesis focusses mainly on the study of triheme cytochromes from *G. metallireducens* (*Gm*) – PpcA, PpcB, PpcC, PpcE and PpcF and *G. sulfurreducens* (*Gs*) – PpcA-E. The cytochromes from *Gm* were characterized at different extent using complementary spectroscopic techniques, namely by UV-visible and NMR. It was concluded that the proteins are structural homologous to those of *Gs*. Cytochrome PpcF was studied in more detail, including the determination of the detailed properties of each heme group. The results obtained revealed that the three hemes exhibit negative reduction potential values that differ from each other and are modulated by a network of redox and redox-Bohr interactions. Furthermore, these values cover a less negative working functional redox window compared to the triheme cytochromes from *Gs*, possibly associated with *Gm*'s capacity to oxidize a broader spectrum of organic and inorganic compounds. Furthermore, the preliminary characterization of cytochrome PpcE revealed unique properties when compared to its homologues from *Gm* and *Gs*.

NMR and UV-visible were also explored to monitor biomolecular interactions and electron transfer reactions involving the well characterized PpcA-E cytochromes from *Gs*. Studies between PpcA and the electron acceptor Fe-NTA showed that the acceptor is reduced by the cytochrome and that the interaction is electrostatically driven by the positively charged lysine residues in the vicinity of the most exposed edge of cytochrome's heme IV. Molecular interactions between each pair of cytochromes showed that the interaction within each pair is transient and unspecific, providing an answer to the question, why the bacteria express constitutively the five cytochromes (PpcA-E). In fact, it was showed for the first time how these small cytochromes regulate the redox potential of the periplasm in a way that the inner membrane, periplasmic and outer membrane cytochromes are at an intermediate oxidation state, *i.e.*, always ready to receive and donate electrons, while assuring a smooth electron transfer from the cytoplasm to the cell exterior.

Keywords: *Geobacter*, extracellular electron transfer, cytochromes, NMR, protein interactions

Resumo

As bactérias *Geobacter* apresentam uma versatilidade respiratória notável, sustentando o seu crescimento através de vários compostos extracelulares, em contraste com os processos respiratórios convencionais que envolvem doadores e aceptadores de eletrões solúveis, o que se reflete num impacto significativo nas aplicações biotecnológicas, como a biorremediação de contaminantes orgânicos e inorgânicos e a produção de bioenergia. Para otimizar estas aplicações, é imperativo compreender o mecanismo de troca de eletrões entre a célula e o ambiente externo, um processo conhecido como transferência extracelular de eletrões (TEE). Os citocromos trihémicos periplasmáticos do tipo *c* foram identificados como componentes essenciais para a TEE, fazendo a ponte entre as proteínas das membranas interna e externa.

Esta Tese centra-se essencialmente no estudo dos citocromos trihémicos de *G. metallireducens* (*Gm*) - PpcA, PpcB, PpcC, PpcE e PpcF e de *G. sulfurreducens* (*Gs*) - PpcA-E. Os citocromos de *Gm* foram caracterizados em diferentes graus utilizando técnicas espectroscópicas complementares, nomeadamente UV-visível e RMN, e concluiu-se que as proteínas são estruturalmente homólogas às de *Gs*. O citocromo PpcF foi estudado em maior detalhe, o que incluiu a determinação das propriedades detalhadas de cada grupo hemo. Os resultados obtidos revelaram que os três hemos apresentam valores de potencial de redução negativos que diferem entre si e são modulados por uma rede de interações redox e redox-Bohr. Além disso, estes valores cobrem uma janela redox funcional menos negativa em comparação com os citocromos trihémicos de *Gs*, possivelmente associada à capacidade de *Gm* para oxidar um espectro mais amplo de compostos orgânicos e inorgânicos. Para além disso, a caracterização preliminar do citocromo PpcE revelou propriedades únicas quando comparado com os seus homólogos de *Gm* e *Gs*.

As espectroscopias de RMN e UV-visível foram também exploradas para monitorizar as interações biomoleculares e as reações de transferência de eletrões envolvendo os citocromos PpcA-E de *Gs*. Os estudos entre o PpcA e o aceptador de eletrões Fe-NTA mostraram que o aceptador é reduzido pelo citocromo e que a interação é electrostaticamente impulsionada pelos resíduos de lisina positivamente carregados na vizinhança da extremidade mais exposta do heme IV do citocromo. As interações moleculares entre cada par de citocromos mostraram que a interação dentro de cada par é transitória e inespecífica, dando uma resposta à questão, por que razão as bactérias expressam constitutivamente os cinco citocromos (PpcA-E). Assim, foi demonstrado pela primeira vez como estes pequenos citocromos regulam o potencial redox do periplasma de forma que os citocromos da membrana interna, do periplasma e da membrana externa se encontrem num estado de oxidação intermédio, ou seja, sempre prontos a receber e a doar eletrões, assegurando ao mesmo tempo uma transferência fluida de eletrões do citoplasma para o exterior da célula.

Palavas chave: *Geobacter*, transferência extracelular de eletrões, citocromos, RMN, interações de proteínas

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ABBREVIATIONS, SYMBOLS AND CONSTANTS

1D	One dimensional
2D	Two dimensional
2xYT	2x yeast extract – tryptone
3D	Three dimensional
AMP	Ampicillin
B ₀	Magnetic field
bp	Base pair
BLAST	Basic Local Alignment Search Tool
Ccm	Cytochrome <i>c</i> maturation
CD	Circular dichroism
CLO	Chloramphenicol
Cryo-EM	Cryogenic Electron Microscopy
Da	Dalton
DIET	Direct Interspecies Electron Transfer
DIPSY	Decoupling in the presence of scalar interactions
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DMSO	Dimethylsulfoxide
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
EDTA	Ethylenediaminetetraacetic acid
<i>E. coli</i>	<i>Escherichia coli</i>
E_{app}/E^0	Macroscopic apparent midpoint reduction potential
e_{app}	Microscopic apparent midpoint reduction potential
EET	Extracellular electron transfer
EXSY	EXchange SpectroscopY
Fe-NTA	Ferric nitrilotriacetate
FID	Free Induction Decay
<i>G. metallireducens</i> /Gm	<i>Geobacter metallireducens</i>
<i>G. sulfurreducens</i> /Gs/GSU	<i>Geobacter sulfurreducens</i>
HCl	Hydrochloride
HMQC	Heteronuclear Multiple Quantum Coherence
HSQC	Heteronuclear Single Quantum Coherence
I	Spin quantum number
Imc	Inner membrane cytochrome
IPTG	Isopropyl β-D-thiogalactoside

IUPAC-IUB	International Union of Pure and Applied Chemistry and International Union of Biochemistry
J	Coupling constant
LB	Luria Bertrani
MFC	Microbial Fuel Cell
MWCO	Molecular Weight Cut Off
NADH	Nicotinamide adenine dinucleotide (reduced form)
NHE	Normal Hydrogen Electrode
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
OD ₆₀₀	Optical Density at 600 nm
Omc	Outer-membrane cytochromes
P	Angular momentum
p_{ijk}	Microstate with hemes i, j, k oxidized
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
pI	Isoelectric point
Ppc	Periplasmic cytochrome
PpcX ^{ox}	Periplasmic cytochrome X in the oxidized form
PpcX ^{red}	Periplasmic cytochrome X in the reduced form
ppm	Parts per million
PSA	Ammonium persulfate
RF	Restriction-free
rpm	Rotations per minute
S _x	Oxidation stage x
SDS-PAGE	Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis
T ₁	Longitudinal relaxation
T ₂	Transverse relaxation
TAE	Tris-Acetate EDTA
TCI	Triple-resonance cryoprobe
TEMED	Tetramethylethylenediamine
TEV	Tobacco Etch Virus
T _m	Melting temperature
TOCSY	Total Correlation Spectroscopy
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
δ	Chemical Shift
γ	Gyromagnetic ratio
ϵ	Molar extinction coefficient
μ	Magnetic moment
τ_m	Mixing period
ν_0	Larmor frequency

F	Faraday constant (96 485 C mol ⁻¹)
<i>h</i>	Planck constant (6.63x10 ⁻³⁴ m ² kg s ⁻¹)
R	Molar gas constant (8.314 J K ⁻¹ mol ⁻¹)
k _B	Boltzmann constant (1.38x10 ⁻²³ m ² kg s ⁻² K ⁻¹)

Amino acid abbreviations

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

| 1.

INTRODUCTION

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1. Introduction

Nowadays, the use of fossil fuels, particularly oil and gas, has accelerated and consequently triggered a global energy crisis. Naturally, research has been directed to the development of alternative and renewable energy sources, in opposition to fossil energy that has major consequences in global warming [1, 2]. Also, the need to guarantee availability and sustainable management of clean water, have been pinpointed dissimilatory metal reducing bacteria as viable alternatives for environmental biotechnological applications.

Dissimilatory metal reduction is a crucial process that plays a fundamental role in the geochemistry of aquatic sediments, submerged soils, and terrestrial subsurface [3, 4]. It is established in major bacterial groups that are capable of coupling their oxidative metabolism to the reduction of metals present in the environment [5]. Unlike other respiratory pathways, dissimilatory metal reduction does not use freely diffusible gas like O₂ or soluble molecules that can be reduced inside the cell. Therefore, it requires the development of an extracellular electron transfer mechanism to send electrons from the cell's interior to the environment, crossing the cellular membrane. This unique mechanism allows electron transfer across a lipidic barrier and influences the biogeochemical cycles of certain metals, such as iron, manganese, uranium, and chromium [3]. Some exoelectrogen organisms can even couple their oxidative metabolism to the electron transfer toward electron surfaces [6].

The extracellular electron transfer (EET) can be achieved by two mechanisms that involve direct or indirect reduction [7, 8]. In the direct transfer, microorganisms physically interact with solid electron donors/acceptors between redox proteins located at the surface of the bacteria or via electrically conductive appendages that have been called biological nanowires [9-12]. In the indirect way, microorganisms (i) synthesize or secrete redox-active compounds that are endogenous electron shuttles, (ii) utilize redox acceptors that are already available in the environment or, (iii) the reduction of electron acceptors is mediated by bacterial-produced organic ligands which are also determined as chelators that solubilize metals before their reduction. The direct transfer is considered to be more advantageous because the synthesis and secretion of redox-active compounds may be costly, and these compounds diffuse and may not be available when needed [13].

Taking advantage of *Geobacter* physiological hallmarks, these bacteria have been used for bioremediation of contaminated waters and energy production in Microbial Fuel Cells (MFC). A MFC is a bioreactor that converts chemical energy in the chemical bonds of organic compounds to electrical energy through catalytic reactions of microorganisms under anaerobic conditions [14]. *Geobacter* species produce higher current densities than any other known organism in MFC and are common colonizers of electrodes harvesting electricity from organic wastes and aquatic sediments [1, 2, 15]. However, currently, the electric current produced by MFC devices is still considerably low for large scale applications [16] and several strategies are being implemented for their improvement such as the design of *Geobacter* strains

with enhanced extracellular electron transfer rates [17, 18]. For this, it is necessary to elucidate these mechanisms which are still poorly understood [19].

Beyond the referred applications of *Geobacter* in renewable bioenergy, the bacterial metabolism can be used for bioremediation of contaminated soils and waters since at physiological pH these bacteria display the particular ability of reducing U(VI), Cr(VI), Tc(VII) and V(V) in contaminated environments to its insoluble forms which promotes the bioremediation of the pollutants [19-21].

The work developed and presented in this Thesis focuses on the functional, thermodynamic, and biochemical characterization of electron transfer components from *Geobacter* bacteria.

1.1 *Geobacter* bacteria

The *Geobacteraceae* family comprises Gram-negative bacteria belonging to the *Desulfuromonadales* order in the δ -proteobacteria class, encompassing four genera of dissimilatory iron-reducing bacteria that do not form spores: *Geobacter*, *Pelobacter*, *Desulfuromonas*, and *Desulfuromusa* [5, 22]. Presently, 19 *Geobacter* species have been isolated from various environments, such as *Geobacter sulfurreducens* (from contaminated ditches), *Geobacter metallireducens* (from aquatic sediments), *Geobacter bemidjiensis* (from Fe(III)-reducing subsurface sediments), *Geobacter lovleyi* (from freshwater sediments), and *Geobacter uraniireducens* (from uranium-contaminated subsurface sediments). The most extensively researched species of *Geobacter* are *G. sulfurreducens* and *G. metallireducens*. While *G. metallireducens* was the first *Geobacter* member to be isolated in 1987 [23, 24], *G. sulfurreducens* is the most well-studied species and is used as a model organism for the *Geobacteraceae* family. *G. sulfurreducens* was only isolated in 1994 from surface sediments of a hydrocarbon-contaminated ditch in Oklahoma, United States of America [25], and its genome was sequenced in 2003 [26], followed by the development of a fully genetic system in 2007 [27].

As previously noted, *Geobacter* has attracted attention for its unique characteristics not found in other living organisms, such as the production of extracellular magnetite from microbial Fe(III) reduction, the use of Fe(III), Mn(IV), or humic substances as electron terminal acceptors in respiration processes, the microbial reduction of water contaminants such as U(VI), Cr(VI), or Co(III), anaerobic oxidation of aromatic hydrocarbons in pure culture, the potential for interaction with syntrophic partners via Direct Interspecies Electron Transfer (DIET), and oxidation of organic compounds to carbon dioxide with an electrode serving as the sole electron acceptor [19]. Usually, *Geobacter* species are found in habitats like aquatic sediments, wetlands, rice paddies and subsurface [19] where Fe(III) is more available as electron acceptor rather than the more common ones such as oxygen, nitrate and sulfate [24, 28]. In fact, *Geobacter* is the predominant microbial community in these environments [25, 29, 30] and is also capable to completely oxidize various organic compounds with diverse electron

acceptors [19, 31] to rapidly populate anaerobic environments [32]. The prevalence of *Geobacter* bacteria in these environments is due to low maintenance energy requirements [33] and the ability to momentarily sustain metabolic activities under starvation conditions [34, 35]. In addition, these are also capable of fixing nitrogen [36] and can thrive under conditions of oxidative stress, as they are supplied with several mechanisms that allow them to survive in such environments [37].

1.2 *Geobacter sulfurreducens* versus *Geobacter metallireducens*

Although *G. metallireducens* was the first to be described, it was only in 2009 that its genome was fully sequenced, which permitted the study of the electron transfer processes and its genetic manipulation [38].

G. metallireducens has the ability to oxidize a larger amount of organic compounds compared to *G. sulfurreducens* species such as aromatic (e.g. benzaldehyde, phenol, benzene and toluene) and non-aromatic (e.g. butanol, ethanol and propanol) [19, 38]. Both *G. sulfurreducens* and *G. metallireducens* has been used for bioremediation of water contaminated with U(VI) and Tc(VII), due to their ability to reduce these compounds. *G. metallireducens* is also able to perform bioremediation of waters contaminated with V(V) [39-41]. Furthermore, the first time a DIET mechanism was reported was in co-cultures of *G. metallireducens* and *G. sulfurreducens* where ethanol was the electron donor and fumarate the acceptor [42]. In this case, DIET was required since *G. metallireducens* could metabolize ethanol but is not able to use fumarate as an electron acceptor, while *G. sulfurreducens* can reduce fumarate but does not metabolize ethanol. *G. metallireducens* can also perform DIET with *Methanosaeta* species in the conversion of wastewaters' organic compounds to methane, an important process used for bioenergy production [43].

To optimize the *Geobacter* based applications it is crucial to better understand the electron transfer mechanisms of these bacteria and in this Thesis contributions to this topic will be presented.

1.3 The most abundant electron transfer proteins in *Geobacter*: *c*-type cytochromes

The *Geobacter* species have more than 100 genes encoding for *c*-type cytochromes, with 85% of these cytochromes containing more than one heme group, as revealed by their sequenced genomes [44]. Cytochromes are proteins that contain iron-porphyrin cofactors, hemes, which are involved in a range of biological functions such as electron transfer, oxygen transport and storage, reduction of peroxides, catalysis, gas sensing, and gene regulation [45]. The versatility of these functions can be enhanced when the hemes are combined with other

co-factors such as flavins and metal ions like molybdenum and copper. The function of these heme-containing proteins is modulated by the type of heme present in the protein and the neighboring amino acids, which define the heme's reactivity, solvent exposure, accessibility to exogenous ligands, distribution of polar and charged groups, axial ligands to iron, and heme-binding site properties [45, 46]. Therefore, heme containing proteins are one of the most studied classes of biomolecules due to their diverse cellular biological functions. Indeed, multiple heme groups confer several advantages to these proteins. Firstly, the presence of more than one heme group enables the protein to transfer more than one electron at a time, and the redox properties can modulate the redox interactions between the hemes and/or by protonable centers in the protein that modulate the potential according to the solution's pH (redox-Bohr effect). Secondly, the collective contribution of the individual heme reduction potential ranges overlaps, extending the protein's global working range. Thirdly, multiple heme groups allow the protein to act as a capacitor, maintaining the bacteria's metabolism during periods when the cells are unable to reduce extracellular electron transfer acceptors [47].

The nomenclature of cytochromes is based on their heme type letter (in italics) and a subscript number that reflects intrinsic characteristics related to protein axial ligand coordination, number of heme groups, and optical or functional properties [48]. In cytochromes *c*, the polypeptide chain binds one or more *c*-type hemes covalently through two thioether bonds between cysteine thiol groups and heme vinyl groups, which are located in a conserved binding motif sequence: CXXCH. The amino acid cysteine is represented by the letter C, X can be any amino acid, and H represents histidine, which acts as the proximal ligand (Figure 1.1) [45, 49]. It is noteworthy that the "XX" segment in the CXXCH sequence is flexible, as all residues except cysteine have been found.

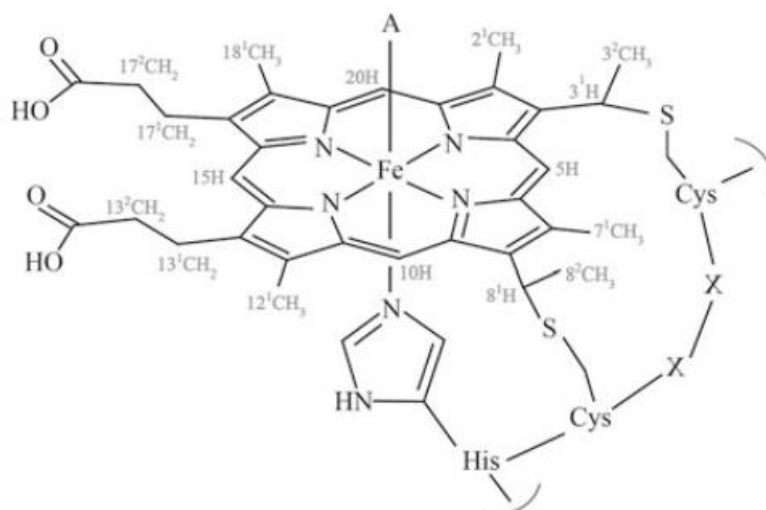


Figure 1.1 - Schematic representation of a c-type heme and the correspondent polypeptide binding motif. The heme molecule has a tetrapyrrole ring system with four nitrogen atoms forming the planar core, which coordinates the iron ion at the center of the heme. The heme also has two axial coordination positions with the proximal position being occupied by the histidine residue (His) of the polypeptide chain. The axial coordination position A can be free or occupied by ligands such as the side chain of methionine, histidine, asparagine, or tyrosine residues, which are the most common. The IUPAC nomenclature for tetrapyrroles is illustrated in gray [50]. This figure was adapted from [47].

The properties of proteins containing heme groups are mostly modulated by the chemistry of the iron atom, which is coordinated by five atoms: four nitrogen atoms of the protoporphyrin IX ring and one nitrogen atom of the imidazole ring of the histidine side chain. The sixth coordination position, also known as the distal ligand, can either be vacant or occupied transiently. In cytochromes with enzymatic activity, the sixth coordination is vacant [48, 51], while in the others it can be occupied by a ligand such as methionine, histidine (in multiheme cytochromes), asparagine, or tyrosine. The axial ligating atom for each of these ligands is different, with nitrogen for histidine or asparagine, sulfur for methionine, and oxygen for tyrosine [48, 49, 51].

The iron (0) is a transition metal with 26 electrons arranged in the electronic configuration $1s^2 2s^2 2p^6 3s^2 3p^6 3d^6 4s^2$, where the numbers indicate the orbitals (s, p and d) and the superscripts indicate electron occupancies. This electronic configuration can also be expressed as $[Ar]3d^6 4s^2$, highlighting the significance of the electron occupancy in the higher orbitals (3d and 4s) in determining the electronic and chemical properties of iron [49]. In electron transfer proteins, the iron is typically found in two oxidation states: the ferrous state (Fe(II): $[Ar]3d^6 4s^0$) and the ferric state (Fe(III): $[Ar]3d^5 4s^0$).

The high and low-spin states are determined by the distribution of electrons in the five 3d orbitals of the iron ion, which include two higher energy orbitals ($d_{x^2-y^2}$ and d_{z^2} , also known as the e_g set) and three lower energy orbitals (d_{xy} , d_{xz} and d_{yz} , also known as the t_{2g} set), according to Crystal Field Theory [52]. Each of these orbitals can hold up to two electrons, and the energy

difference between them is influenced by the ligands coordinating the iron ion. The distribution of electrons in the d-orbitals is governed by two main energy terms: the strength of the d-orbital splitting (represented by Δ_0) and the energy required to pair electrons in the same orbital (pairing energy - P) [49]. If the d-orbital splitting is smaller than the pairing energy (weak crystal field), the iron ion is in the high-spin state, with mostly unpaired electrons in the e_g and t_{2g} orbitals. On the other hand, if the d-orbital splitting is larger than the pairing energy (strong crystal field), the electrons pair in the t_{2g} orbitals, resulting in the low-spin state (Figure 1.2). The porphyrin ring is a strong ligand that positions the iron ion near a state where small energy variations in the axial ligand field can prompt a change in the spin state [49]. The presence of weak crystal field is observed when heme iron binds to an asparagine, lysine, tyrosine, or water molecule, while a strong crystal field is typical of methionine or histidine as axial ligands [53]. Determining the spin state of a heme protein is crucial to obtain information about the nature of the heme axial ligands. A range of spectroscopic techniques, such as Nuclear Magnetic Resonance (NMR), UV-visible, and Electron Paramagnetic Resonance (EPR), can be explored to investigate the heme spin state [54].

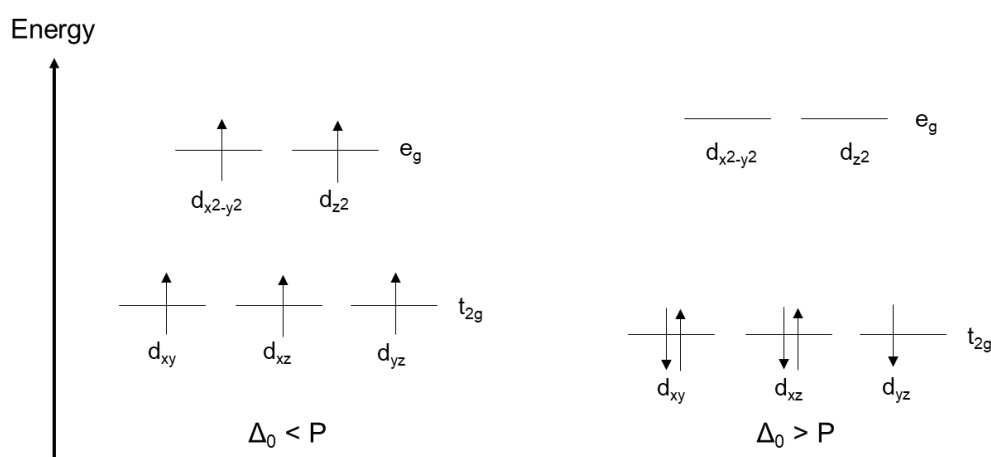


Figure 1.2 - Spin-states of octahedral Fe(III). When placed in an octahedral field, 3d-orbitals of the iron atom are rearranged in two sets the t_{2g} set (of lower energy, composed by the d_{xy} , d_{xz} and d_{yz} orbitals) and the e_g set (of higher energy, composed by the $d_{x^2-y^2}$ and d_{z^2} orbitals). A weak crystal field ($\Delta_0 < P$) leads to a total spin of 5/2 (high-spin configuration), whereas a strong crystal field ($\Delta_0 > P$) results in a total spin of 1/2 (low-spin configuration).

1.4 Extracellular electron transfer in *Geobacter*

The genome of *G. sulfurreducens* encodes for 73 multiheme cytochromes [55]. As previously mentioned, the main advantage of having a high content of multiheme cytochromes is to provide the cell with a high electron storage capacity. Moreover, these cytochromes allow electron transfer through large distances without various physiological partners and binding events. Typically, the heme groups of these cytochromes are arranged along the polypeptide chain, with no more than 16 Å distance between them, permitting a fast electron exchange [7, 51]. Multiheme cytochrome typically present His-His coordination making its redox potential usually lower than monoheme cytochromes where a His-Met coordination is more frequent [56].

Geobacter cytochromes are strategically located at different parts of the bacterial cell, such as the inner membrane (IM), periplasm, or outer membrane (OM), to facilitate extracellular electron transfer (EET). This process enables the transfer of electrons from intracellular carriers to extracellular acceptors. Among *Geobacter* species, *G. sulfurreducens* has been extensively studied regarding its EET mechanisms, due to the early development of a genetic system that allows for proteomic and gene-knockout studies.

The transfer of electrons from the oxidation of organic compounds in *G. sulfurreducens* is hypothesized to occur via a NADH hydrogenase located in the bacterial inner membrane [47], according to the most recent model for EET presented in Figure 1.3. Subsequently, depending on the redox potential of the final electron acceptor, the electrons follow one of three pathways: the CbcBA pathway when the final electron acceptors have redox potentials below -210 mV (vs. the normal hydrogen electrode, NHE), the CbcL-dependent pathway, which is activated when the redox potential of the acceptor is between -210 and -100 mV, or the ImcH pathway when the redox potential is above -100 mV [57-59]. The electrons are then transported from CbcBA, CbcL or ImcH to periplasmic cytochromes (mainly to the PpcA-family) and subsequently to porin-cytochrome trans-outer membrane complexes which carry the electrons to extracellular electron acceptors [60].

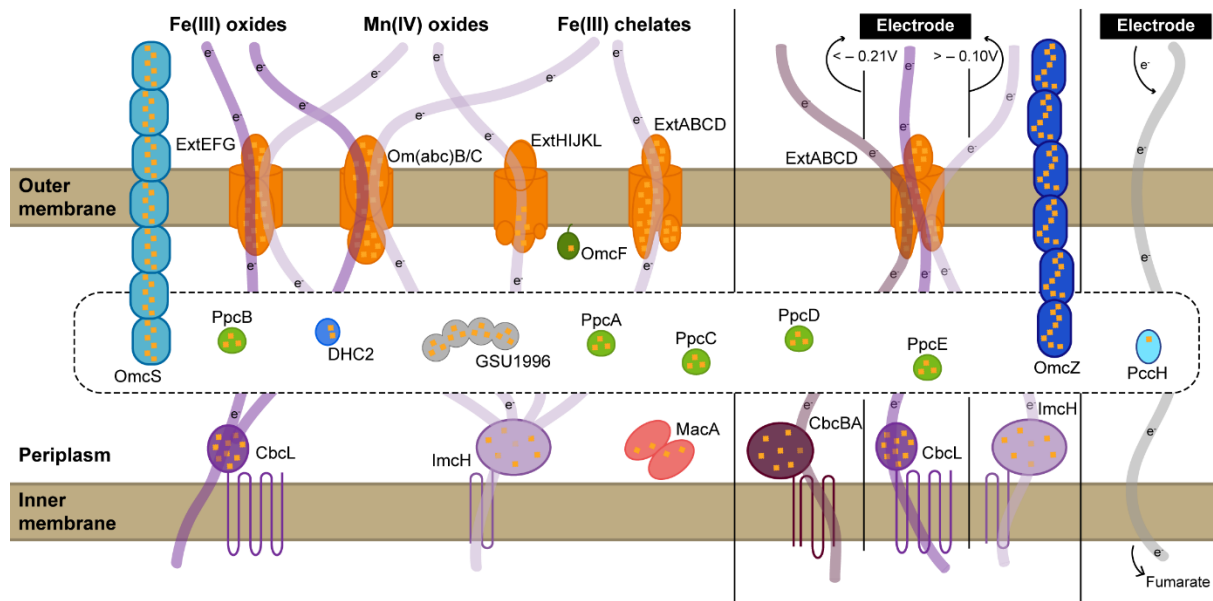


Figure 1.3 - Model for the extracellular electron transfer pathway of *G. sulfurreducens*. The different c-type cytochromes responsible for electron transfer pathways involving Fe (III) oxides, Mn(IV) oxides, Fe(III) chelates, as well as electrodes, are depicted along with their putative direction of electron flow. CbcBA, CbcL and ImcH are proteins associated with the inner membrane that have been demonstrated to play a crucial role in the reduction of extracellular electron acceptors with reduction potential values below -210 mV, between -210 and -100 mV and above -100 mV, respectively [57-59]. There are five distinct outer membrane complexes responsible for facilitating electron transfer to the cell exterior: ExtABCD, ExtEFG, ExtHIJKL, Om(abc)B, and Om(abc)C. A comprehensive study involving the deletion of genes encoding individual complexes or combinations thereof demonstrated that different electron transfer pathways involve distinct complexes [61]. Several proteins have been identified within the periplasm, but their precise roles in specific electron transfer pathways remain uncertain. Proteins shown to interact in vitro are connected by a straight arrow [62, 63]. The c-type heme groups are represented in light orange. Adapted from [64].

In addition to these electron transfer proteins, *Geobacter* bacteria hold electrically conductive pili that establish electrical contacts with electrodes and other methanogenic species, and flagella that allow them to move toward solid extracellular electron acceptors [65]. Recently, it was discovered that some of the extracellular cytochromes, namely OmcS, OmcZ and OmcE are capable of polymerizing and forming electrically conductive filaments, illustrating the vast complexity of these mechanisms [66-70].

To date, the mechanisms of EET in *G. metallireducens* are considerably less studied but given the fact that this strain can reduce an even greater number of electron acceptors, there has been an increasing interest in comprehending the electron transfer mechanisms in *G. metallireducens*. Present studies point to the conservation of some electron transfer components, such as the periplasmic cytochromes of the PpcA-family and the outer membrane cytochromes OmcB and OmcE, but further structural and functional information on the elucidation of these pathways are needed [71-74].

1.5 The PpcA-family

The PpcA family is composed by triheme cytochromes, belonging to the family/sub-class *c7*. In this group of cytochromes, the three heme groups have low reduction potentials (Table 1.1) and are bishistidinyll axially coordinated. The hemes are covalently attached to the polypeptide chain by the CXXCH motifs [49]. Each heme is low-spin both in the reduced diamagnetic state (Fe(II), $S=0$) and in the oxidized paramagnetic state (Fe(III), $S=1/2$) which gives rise to specific features in the UV-visible spectrum (Figure 1.4). This family is one of the most conserved among *Geobacter*, with multiple homologs in most of the genomes: five in *G. sulfurreducens*, five in *G. metallireducens*, four in *G. uraniireducens*, three in *G. bemidjensis*, and one in *G. lovleyi* [44]. In *G. sulfurreducens*, the PpcA-family is composed by five homolog cytochromes, designated PpcA, PpcB, PpcC, PpcD, PpcE.

Table 1.1 - Heme reduction potentials values and pairwise interactions (mV) of the fully reduced and protonated forms of PpcA, PpcB, PpcD, PpcE of *G. sulfurreducens* (Gs) and PpcA of *G. metallireducens* (Gm) [71, 75]. The reduction potentials values are relative to NHE.

Cytochrome	Reduction potential values of the hemes			Redox interactions			Redox-Bohr interactions		
	I	III	IV	I-III	I-IV	III-IV	I-H	III-H	IV-H
PpcA Gs	-154	-138	-125	27	16	41	-32	-31	-58
PpcB Gs	-150	-166	-125	17	8	32	-16	-9	-38
PpcD Gs	-156	-139	-149	46	3	14	-28	-23	-53
PpcE Gs	-167	-175	-116	27	5	22	-12	2	-13
PpcA Gm	-80	-70	-113	35	3	37	-22	-23	-49

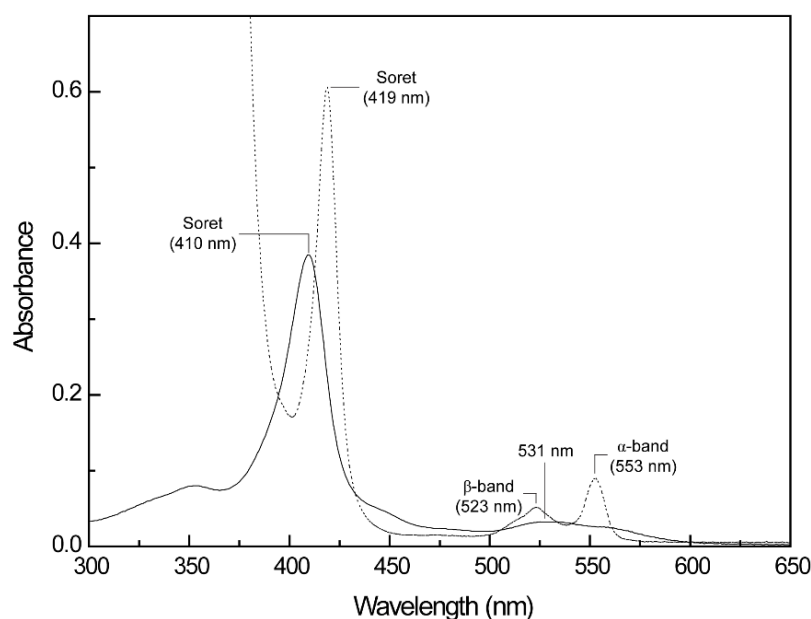


Figure 1.4 - UV-visible spectra of PpcF from *G. metallireducens* in the fully oxidized (solid line) and fully reduced (dashed line) states. The characteristic bands of the spectra in both redox states are indicated.

The PpcA-family members are highly abundant in the cell, and it is proposed that they function as electron carriers between the inner and outer membrane. Structural studies have suggested that PpcA and its homologs may interact with common redox partners, however they are not always simultaneously present and their abundance can also vary [75-77]. Primary proteomic studies demonstrated that PpcA, PpcB and PpcD were present in cell cultures grown in Fe(III) citrate and Fe(III) oxides, whereas PpcE was only found in cultures grown in the presence of Fe(III) citrate [78]. Knockout studies where *ppcA* gene was deleted demonstrated that *G. sulfurreducens* reduced Fe(III) citrate at rates 58 % of those of wild-type [79], suggesting that PpcA is an important, however not essential, element for EET. Also, *ppcB* and *ppcC* knockout mutants showed decreased growth in Fe(III) citrate but not in Fe(III) or Mn(IV) oxides [78]. On the other hand, in the presence of Fe(III) citrate, both *ppcD* and *ppcE* knockout mutants increased Fe(III) reduction and cell growth rate. However, *ppcA* and *ppcD* genes were upregulated in growth in Mn(IV) oxides when compared to Fe(III) citrate [80]. Novel studies using strains where the five homologs genes were deleted demonstrated that these were unable to reduce Fe(III), while strains only functional for PpcA were sufficient for the cells to reduce Fe(III) citrate at rates comparable to the wild-type strain [81]. These apparently conflicting results hint that in the single knockouts, the other homologs may contribute to the adaptation of the strains.

While in *G. sulfurreducens*, PpcA-family is composed by PpcA-E, in *G. metallireducens* the family is constituted by PpcA, PpcB, PpcC, PpcE and PpcF. The PpcF designation results from its lowest amino acid sequence identity with cytochrome PpcD from *G. sulfurreducens* (Table 1.2). The members of the two families are composed of about 70 amino acids with a molecular mass of approximately 10 kDa and a pI of 9 [72, 73, 75]. The sequence alignment, as well as percentage of identity relative to PpcF are indicated in Figure 1.5.

Table 1.2 - Amino acid sequence identity (%) between the PpcA-family from *G. sulfurreducens* and *G. metallireducens*. The percentage of identity was obtained from the basic local alignment search tool (BLAST) [82].

	<i>G. sulfurreducens</i>					
	A	B	C	D	E	
<i>G. metallireducens</i>	A	80	73	64	68	62
	B	68	72	57	68	63
	C	59	56	79	42	51
	E	54	61	52	55	69
	F	62	58	57	55	57

In *G. metallireducens*, the PpcA family is known to be crucial for Fe(III) and U(VI) reduction [79] and the *ppcA* gene is generally up-regulated in DIET processes of co-cultures of *G. metallireducens* with other organisms such as *G. sulfurreducens* or *Methanosarcina barkeri* [83, 84].

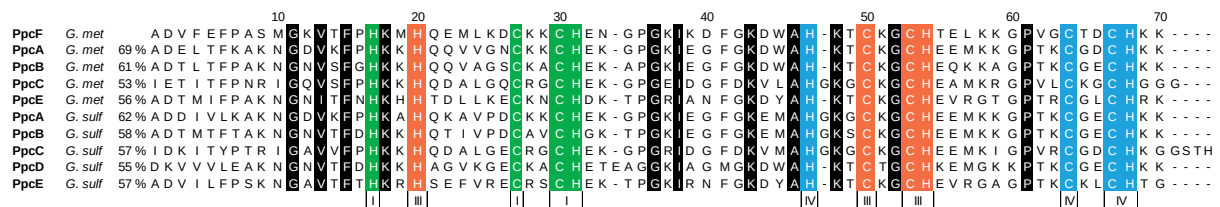


Figure 1.5 - Alignment of the amino acid sequences of the periplasmic triheme cytochromes from *Geobacter sulfurreducens* and *Geobacter metallireducens*. The conserved residues in the proteins are boxed and colored (green, orange, and blue for hemes I, III, and IV, respectively) and black, for the heme-attached and non-heme-attached, respectively. The heme numbering and the respective attached residues are indicated at the bottom of the last cytochrome amino acid sequence. The percentage of sequence identity relative to PpcF from *G. metallireducens* obtained from the basic local alignment search tool (BLAST) [82] is also indicated.

1.6 Bacterial electrically conductive filaments

As previously mentioned, *Geobacter* bacteria were selected as preferential targets for bioremediation of contaminated environments and for current production in microbial fuel cells. The capability of these bacteria to perform EET relay on the unusual localization of their respiratory electron transfer components at the bacterial outer membrane in addition to the more common localization at inner membrane and periplasm as found for non-exoelectrogens microorganisms, allowing the transfer of electrons from intracellular carriers to extracellular acceptors (Figure 1.3).

G. metallireducens is one of the most effective Fe(III) oxide-reducing *Geobacter* species [23], is able to establish DIET with methanogenic microorganisms [43, 83] and produces high levels of current in microbial fuel cells [38, 85]. All these features were attributed to the presence of pili filaments [85]. Pili, also referred by some authors, as bacterial nanowires, are polymeric assemblies of PilA subunits (6.6 kDa), composed by 59 amino acids with several aromatic residues in positions conserved throughout the *Geobacter* family. These aromatic residues were thought to be responsible for electron transfer since point mutations, replacing them by non-aromatic residues, have affected the EET [86]. Therefore, the cellular location of the pili, running from the entire periplasm, through the outer membrane and expanding to the extracellular space (Figure 1.6), indicates that they are likely to receive electrons from the most abundant periplasmic cytochromes, as it is the case of the members of the PpcA-family.

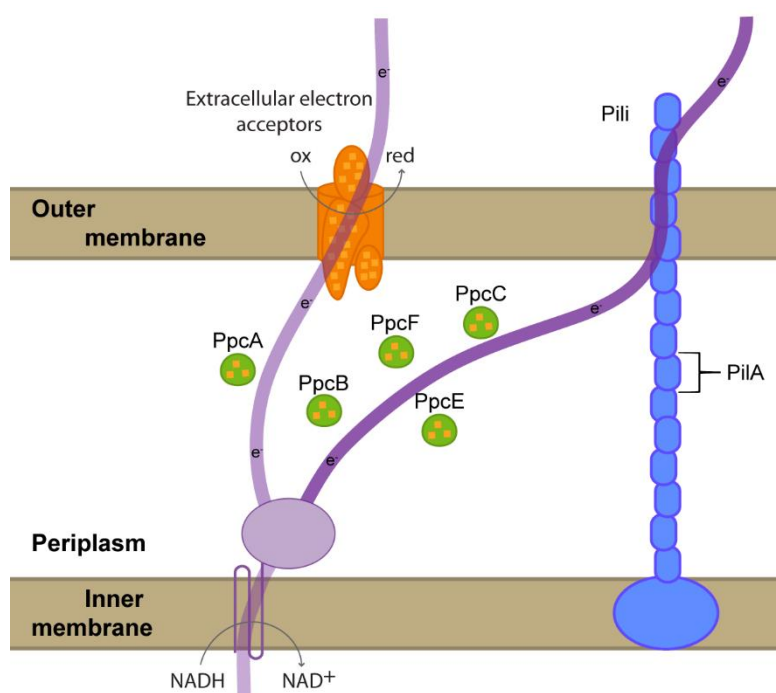


Figure 1.6 - Schematic view of the extracellular electron transfer pathways in *G. metallireducens*. Periplasmic triheme cytochromes PpcA, PpcB, PpcC, PpcE and PpcF are represented in green. Pili (composed by PilA subunits) is represented in blue. The darker electron pathway represents the one proposed to be study in this Thesis.

However, during the time course of this Thesis, cryo electron microscopy (cryo-EM) has identified several bacterial filaments formed by polymerized cytochrome units in *Geobacter sulfureducens* [67, 68, 87]. The first report of a conductive bacterial filament formed by the polymerization of *c*-type cytochrome subunits was reported for OmcS [67]. In this case, each monomer (47 kDa) contains six *c*-type low-spin hemes coordinated by two axial histidines [67]. In the filament, all hemes from one monomer are coordinated by their own axial histidine except the fifth heme that coordinates to a histidine from a neighboring monomer. Later, filaments formed by the polymeric assembly of *c*-type cytochromes OmcZ monomers (32 kDa; eight low-spin hemes) [88] and OmcE (26 kDa; four hemes) [89] were also identified and their structures were determined by Cryo-EM [68, 87]. These proteins are also present in *G. metallireducens*, and the structures have shown that all the hemes are also His-His coordinated [68, 87]. Like in OmcS filament, in the OmcE the heme coordination involves histidine residues from neighboring subunits in adjacent positions. However, in the case of OmcZ the filament arrangement is distinct from the heme packing seen in OmcS and OmcE. Furthermore, in each OmcZ subunit, all eight hemes are axially coordinated by histidines from the same subunit [87]. The structure of these filaments is indicated in Figure 1.7. Overall, these findings have challenged the long-held belief since 2005 [90] and evidence has been collected that the type IV pili function as secretory systems of the polymeric forms made from *c*-type cytochrome units [91].

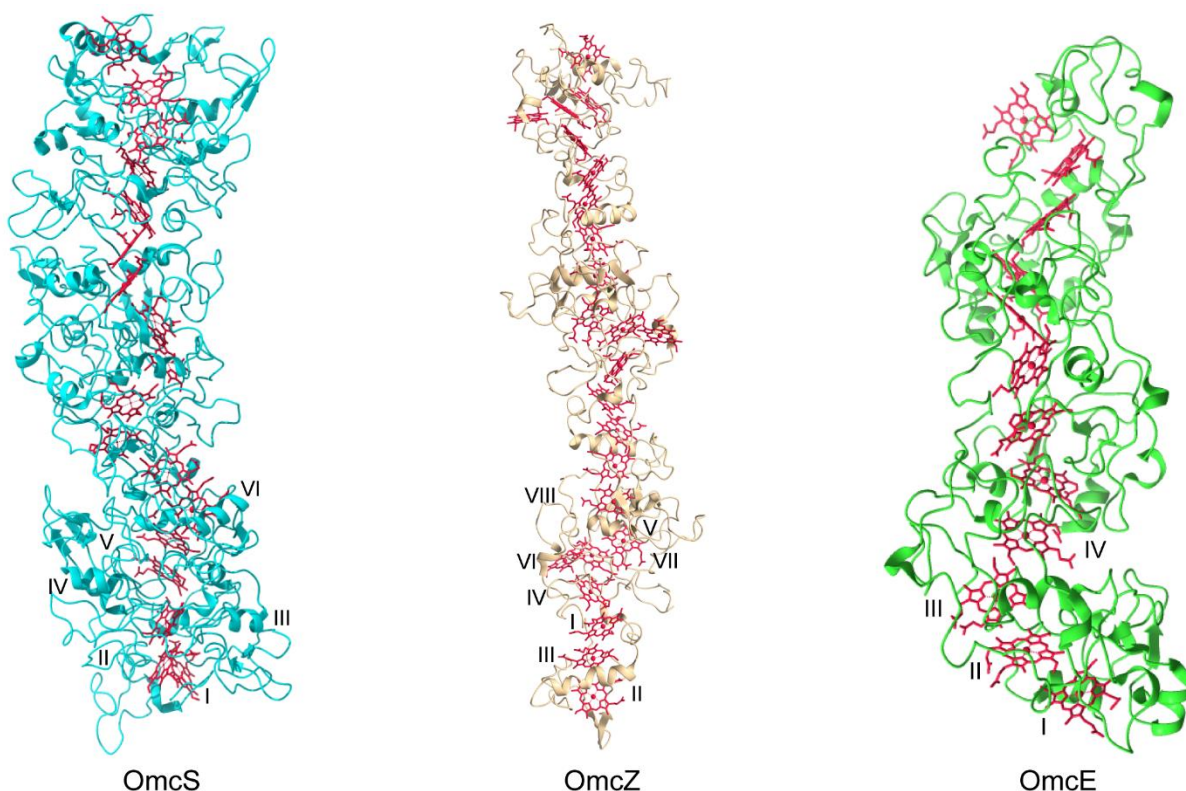


Figure 1.7 – Cryo-EM structures of *G. sulfurreducens* filaments. The structure of cytochromes OmcS (PDB code 6EF8 [67]), OmcZ (PDB code 8D9M [87]) and OmcE (PDB code 7TFS [68]) is represented. Roman numerals indicate the hemes.

Pili have been thought to be composed solely by PilA protein, namely PilA-N [90]. However Cryo-EM revealed that C-terminal residues of PilA-N stabilize its copolymerization with PilA-C (to form PilA-N-C) through electrostatic and hydrophobic interactions that position PilA-C along the outer surface of the filament [91]. It was also collected evidence that these pili remain confined to the periplasmic space under conditions where nanowires formed by the assembling of cytochrome subunits are produced and EET is required [91, 92]. In addition, the deletion of *pilA-N* results in the cessation of the secretion of the bacterial nanowires OmcS and OmcZ. This secretion is restored after the reconstitution of PilA-N-C filaments [91]. Furthermore, if *pilA-N* is substituted with type IV pili from other microorganisms, it also leads to the loss of OmcZ nanowire secretion. Thus, from these observations it was concluded that the disruptive effects in EET attributed to pili were in fact caused by perturbations on the OmcZ's secretory system (pili) and not because they are directly involved in these pathways. Also, the conductivity of PilA-N-C filaments is found to be 20,000 times lower compared to that of OmcZ nanowires [91], which will not explain the high EET rate (10^6 s^{-1}) to electrodes [93].

Hence, based on the recent studies revealing the association between the PilA protein and their role on the secretion of nanowires made of the polymeric assembly of *c*-type cytochromes there is no rationale to study the interactions between the PilA protein from *G. metallireducens* and the periplasmic cytochromes, which was one of the initial goals of this Thesis.

1.7 Triheme cytochromes structural characterization

The determination of multiheme cytochromes structures is crucial to understand their functional mechanisms. In the last decades structures of the triheme cytochromes from *G. sulfurreducens* were determined by NMR and X-ray crystallography. The diffraction data obtained by X-ray crystallography represent an average over molecules arranged in a periodic crystal lattice whereas the structures obtained by NMR represent the average over oriented molecules in solution, which conditions are more similar to the physiological environment of the biomolecules. Furthermore, the solution structure provides information about the internal motions and dynamics of the protein and allows determining interaction regions with possible physiological partners.

However, due to the numerous proton-containing groups of the hemes in cytochromes and the magnetic properties of the heme iron, the polypeptide chain signals are broadened and deviated from their typical position in the NMR spectrum, a feature that is especially pronounced in the oxidized state [75, 94, 95]. These aspects make the assignment of the NMR signals arduous and consequently the structure determination. Further, to acquire NMR data to assist the structure determination, proteins must be isotopically labeled, a methodology that has been improved lately but is still a limiting step due to the low production yields. Therefore, most of the cytochrome's structures deposited in the Protein Data Bank (PDB: <http://www.rcsb.org/pdb/>) were determined by X-ray crystallography, and the ones determined by NMR were mostly acquired in the reduced state.

As mentioned, the structures of the triheme cytochromes from *G. sulfurreducens* has been determined and are indicated in the Figure 1.8. The structures show conserved motifs: a two-strand β -sheet segment at the N terminal, followed by several α -helixes located in different regions of the protein [77]. The spatial arrangement of the hemes in these cytochromes is conserved, with heme III nearly perpendicular to both hemes I and IV [77]. In fact, hemes' designation from the PpcA-family as *c₇* triheme cytochromes derives from the heme spatial arrangement which is superimposed with those of the structurally homologous tetraheme cytochromes *c₃* in which the formers lack heme II and the correspondent polypeptide segment, hence the hemes are numbered I, III and IV [96, 97].

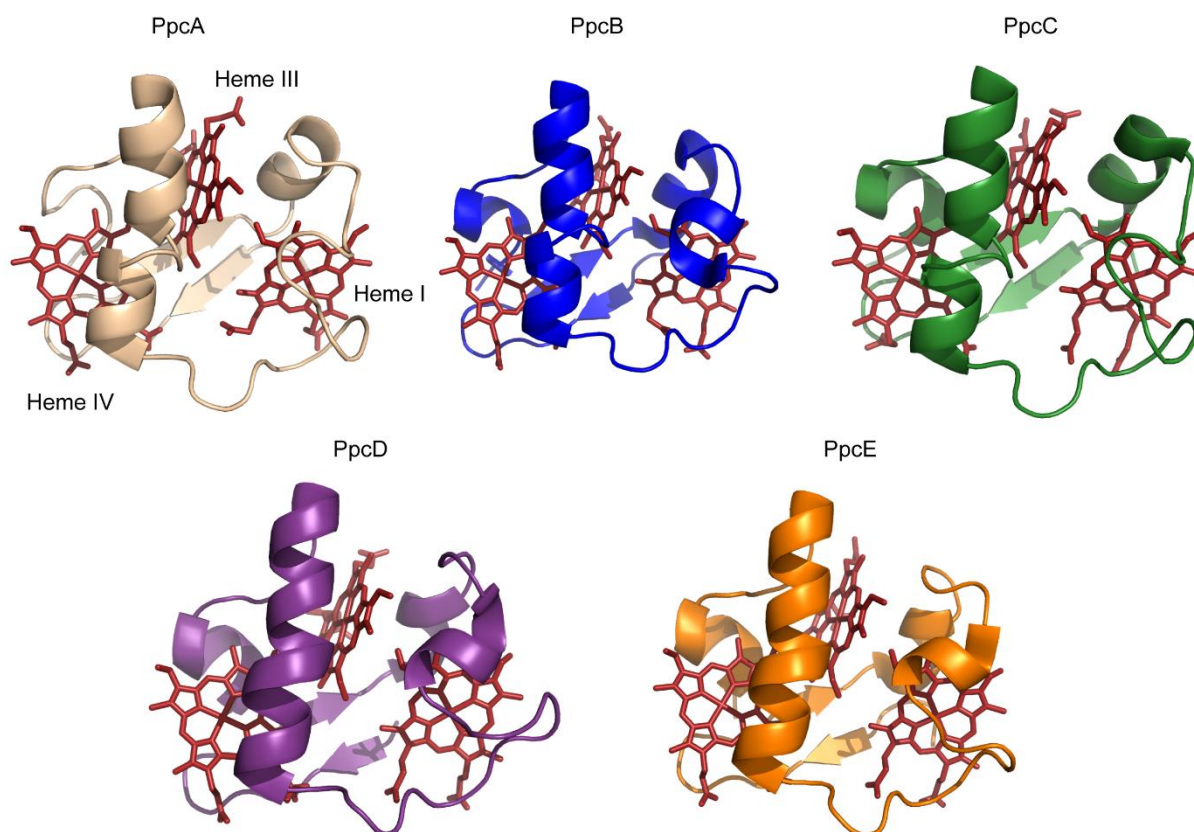


Figure 1.8 - Structures of PpcA family members from *Geobacter sulfurreducens*. PpcA is represented by its solution structure in the reduced state (PDB code 2LDO [98]) and PpcB (PDB code 3BXU [99]), PpcC (PDB code 3H33 [77]), PpcD (PDB code 3H4N [77]) and PpcE (PDB code 3H34 [77]) are represented by their crystal structures. The molecules are all in the same orientation. Figure produced with PyMOL [100].

In this family, heme III is the least exposed in contrast with heme I that is the highest exposed heme. Heme IV shows the largest positive electrostatic surface due to the considerable number of neighboring lysine residues, except for PpcE. The most conserved region between the family members is around heme IV, and the least conserved one around heme I [77].

1.8 Objectives and Thesis outline

The main goal of this Thesis is to contribute to the elucidation of the EET processes in *Geobacter*. Step by step such elucidation can be explored in the future to improve bioremediation and biotechnological applications.

The targeted proteins in this Thesis include members of the PpcA-family from *G. sulfurreducens* and *G. metallireducens*. The thesis also extends to the PilA protein, which is the structural subunit of the bacterial filament Pili. Given the heterogenous group of the targeted proteins, the main goal of this Thesis can be divided in different sub-goals. For the members of the PpcA-family from *G. metallireducens* the goal was the improvement of their heterologous expression in *E. coli* followed by their biochemical characterization, including the

determination of their functional properties and structural features. In the case of the PilA protein, the main goal was its production for the first time in our lab to assist interacting studies with putative partners. Finally, for the members of the well-studied PpcA-family from *G. sulfurreducens*, the main goal was to move a step forward and study biomolecular interactions between these cytochromes and possible redox partners.

This Thesis is divided in seven Chapters. A general introduction was presented in this chapter. Chapter 2 describes the fundamentals of NMR and thermodynamic characterization of multiheme cytochromes. Chapter 3 focus on the improvement of the expression and purification protocols of the triheme cytochromes PpcB, PpcC and PpcE from *G. metallireducens*. The preliminary biochemical characterization of these proteins is also described in this Chapter. The functional and structural characterization of cytochrome PpcF from *G. metallireducens* is presented in Chapter 4. Chapter 5 focuses on experimental description of the production of the PilA protein from *G. metallireducens*. Chapter 6 is dedicated to the biomolecular interaction studies carried out with the members of PpcA-family from *G. sulfurreducens* using NMR. The Chapter is divided in two parts and describes the interaction studies between PpcA from *G. sulfurreducens* and the electron acceptor Fe-NTA, as well as the interactions between the five members of this family. Finally, Chapter 7 presents an overall discussion on the results obtained together with some concluding remarks and future research perspectives.

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**FUNDAMENTS OF NUCLEAR MAGNETIC
RESONANCE AND ITS APPLICATIONS IN THE
CHARACTERIZATION OF MULTHEME
CYTOCHROMES¹**

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2. Fundamentals of Nuclear Magnetic Resonance and its applications in the characterization of multiheme cytochromes

2.1 Fundamentals of Nuclear Magnetic Resonance

NMR spectroscopy is a very powerful technique that has been widely used in structural biology providing information about protein structure, dynamics and interaction with other proteins or ligands.

In general terms, NMR spectroscopy measures the absorption of electromagnetic radiation of a specific frequency by an atomic nucleus placed in a strong magnetic field. In fact, nuclei have positive charges, and many behave as little spin bars. In fact, the movement of charged particles originate a magnetic moment (μ) and produces a magnetic field (B). Therefore, a spinning nucleus acts as a magnet oriented along the spin rotation axis, called nuclear spin. An external magnetic field aligns nuclear spins into specific energy levels. Each energy level is populated by the spins which have the same angular momentum, and the most favorable will be the low-energy state.

The angular momentum, and the magnetic moment of a nucleus are related by the gyromagnetic ratio, γ , (Equation 1) which is characteristic of each nucleus.

$$\mu = \gamma P \quad (1)$$

Nuclei are characterized by a quantum spin number (I), which can be determined by its atomic mass. When $I = 0$, there is no nuclear spin, and it is NMR silent. Most nuclei of biologic interest (e.g. ^1H , ^{13}C , ^{15}N , ^{19}F and ^{31}P) have nuclear spin quantum $I = \frac{1}{2}$. The value of the quantum spin number determines the number of energy spin state levels of a nucleus in presence of an external uniform magnetic field in accordance with the formula $2I+1$. For a nucleus with $I = \frac{1}{2}$, in the absence of an applied magnetic field (B_0) the two energy states are degenerated and the number of nuclei in each state will be equal to the thermal equilibrium. On the other hand, in the presence of an external magnetic field the energy difference (ΔE) between the energy states α ($m_I = +\frac{1}{2}$, oriented with the external magnetic field) and β ($m_I = -\frac{1}{2}$, against the orientation of the external magnetic field) gives rise to the frequency of the spectra, whereas intensities of the signals are proportional to the population difference between α and β states. The ratio of the populations in the states is quantitatively described by the Boltzmann equation (Equation 2):

$$\frac{N_\alpha}{N_\beta} = e^{\Delta E/k_B T} \quad (2)$$

where N_α and N_β represents the number of nuclei in each possible spin orientation, k_B the Boltzmann constant and T the temperature. There will be an excess of nuclei in state α because it is the less energetic one, leading to a net nuclear magnetic moment commonly known as magnetization (M), represented by a vector parallel to the static magnetic field B_0 .

The necessary energy to induce transitions between the states depends on the magnetic field (B_0) that is applied to the nuclei, designated as the nuclear Zeeman splitting, and must equal the ΔE value, as described by Equation 3 where h represents Planck's constant:

$$\Delta E = \frac{\gamma h B_0}{2\pi} \quad (3)$$

In the presence of an external magnetic field B_0 , nuclei have an intrinsic frequency, which is known as the Larmor frequency (ν_0), related to B_0 by Equation 4.

$$\nu_0 = \frac{\gamma B_0}{2\pi} \quad (4)$$

Molecules are subjected to a strong external magnetic field, however their nuclei are surrounded by moving electrons from the chemical bonds that generate a local magnetic field, B_{local} . Consequently, nuclei will have a specific Larmor frequency, which slightly differs from the Larmor frequency expected only when considering the B_0 effect. Hence, not all the nuclei are subject to the same chemical environment. Because of these slight differences nuclei of the same nature in different chemical environments will possess different ν_i and therefore will not be equivalent, which is reflected in a different chemical shift (δ), expressed in ppm (parts per million), and can be distinguished by NMR. The resonance frequencies are expressed in terms of an empirical quantity called chemical shift, which is related to the difference between the resonance frequency (ν_i) of a nucleus in question and that of a reference standard (ν_{ref}), Equation 5:

$$\delta = \frac{\nu_i - \nu_{\text{ref}}}{\nu_{\text{ref}}} \cdot 10^6 \quad (5)$$

The approach to any structural or mechanistic problem will invariably start with the acquisition of one dimensional (1D) spectrum since it provides the foundations to plan the subsequent work. In the NMR vector model, a 1D experiment encompasses two periods: preparation and detection. In the preparation period, a 90° radiofrequency pulse is applied, which rotates the magnetization equilibrium, M , from the z axis (aligned with B_0) to the xy plane. During the detection period, the magnetization will return to the equilibrium (z axis) and its loss along the xy plane will be detected in the form of a free induction decay (FID). A plot of the frequencies of the nuclei *versus* the signal intensities constitutes the 1D NMR spectrum. While in the case of small molecules the 1D spectrum can be sufficient to obtain the required information, for a macromolecule it is too complex to be interpreted. To improve the spectral resolution, an additional dimension can be introduced to disperse the signals over two (2D) or more frequency dimensions (typically 3D) yielding multi-dimensional NMR spectra.

In the case of 2D NMR spectra, they include one additional period called the evolution time, which contains a variable time delay t_1 , and is introduced between the preparation and acquisition periods. Moreover, the second dimension can be frequency for ^1H , which results

in a square spectrum with diagonal peaks, or a heteronuclear acquisition, which gives asymmetric spectra. 2D experiments can also contain other periods in addition to the evolution time, such as a mixing time, t_m . At the end of each experiment, all the FIDs are transformed with the same phase parameters. The transformation of the second FID obtained by the t_1 evolution time generates a second frequency domain that results in a 2D spectrum in which the third dimension corresponds to the signal's intensity.

2.2 NMR experiments

To characterize a protein structurally and functionally, sets of NMR experiments are required. The 1D and 2D experiments used in this Thesis are summarized in Table 2.1.

Table 2.1 – 1D and 2D NMR experiments used in this Thesis. Black and white rectangles represent 90° and 180° pulses, respectively. The FID represents the data acquisition periods. The time delay is represented as t_1 and t_2 , and the mixing time as t_m . Pre-acquisition delay is represented as d_1 , and d_2 is optimized to be $\frac{1}{4} J$ in the HSQC. DIPSI-2 is a pulse sequence designed for isotropic mixing purposes in 2D ^1H -TOCSY. Adapted from [1, 2].

NMR Experiment	Pulse sequence
1D ^1H	
2D ^1H -TOCSY	
2D ^1H -NOESY / 2D ^1H -EXSY	
2D ^1H ,X-HSQC	

In order to assign the heme groups resonances, it is necessary to acquire the following spectra: 2D ^1H -TOCSY and 2D ^1H -NOESY.

2D ^1H -TOCSY (Total Correlation SpectroscopY) experiment uses scalar coupling (through bond nuclear interactions) to correlate all protons within a given spin system. Using J-coupling, magnetization is transferred along the side chain during the mixing time not only

for nuclei which are directly coupled but also between nuclei which are connected by a chain of couplings providing geometric information about molecules [3, 4]. By adjusting the mixing-time in this experiment it's possible to change the proportion of the magnetization transfer. Longer mixing-times (80 to 120 ms) will result five- or six-bond transfers, whereas short mixing-times (20 ms) will result in one-bond transfers [4].

2D ^1H -NOESY (Nuclear Overhauser Effect Spectroscopy) experiment relies on the Nuclear Overhauser Effect (NOE) to exchange magnetization between protons that have spatial proximity (up to 5 Å). NOE is defined as the change in intensity of one NMR resonance as a consequence of the saturation of another close by resonance, which is a result of dipole-dipole cross-relaxation during the t_m period and the resulting resonance intensities are inversely proportional to the sixth power of the distance (r^{-6}) between the interacting spins [3]. Hence, this experiment is crucial to obtain structural restraints for solution structure calculations, whilst it is also needed for specific assignments [4]. The 2D ^1H -EXSY (EXchange Spectroscopy) experiment is composed by the same pulse sequence of the NOESY experiment. However, this experiment is mainly used to investigate cross-relaxation due to chemical exchange, making it essential to study cytochromes in intermediate states of oxidation. Nuclei that have not undergone exchange will resonate at the same characteristic frequencies f_1 and f_2 will lie along the diagonal of the 2D spectrum. Nuclei that have undergone exchange will have different frequencies in f_1 and f_2 and will lie off the diagonal. The magnitude of these cross-peaks is directly related to the exchange rate [5]. In these experiments experimental conditions such as the magnetic field strength, temperature and ionic strength must be optimized in order to obtain exchange rates that match to the EXSY timescale (1 to 10^{-3} seconds) [5-9].

To assign the resonances of the protein's backbone, 2D ^1H , ^{15}N -HSQC and a set of 3D spectra are required, namely HNCA, HN(CO)CA, HNCACB and HN(CO)CACB.

2D ^1H ,X-HSQC (Heteronuclear Single Quantum Coherence) experiment correlates single-bonds between ^1H and a nucleus X (e.g., ^{15}N or ^{13}C). In this, a first pulse sequence generates single quantum coherences between ^1H and its attached nucleus, X, and the magnetization of both nuclei progress during t_1 . Afterward, a second pulse sequence transfer the magnetization back to ^1H for detection [1, 3].

Three-dimensional (3D) NMR experiments derive from the 2D pulse sequences with the addition of additional evolution time (t_2) and mixing-time between the first mixing period and the acquisition.

In 3D HNCA experiment, at first, the magnetization is passed from ^1HN to ^{15}N and then, via N- C_α J coupling, to the $^{13}\text{C}_\alpha$. Then, the magnetization is passed back to ^{15}N and ^1HN respectively before detection of the signal (Figure 2.1A). The chemical shift evolves for ^1HN , as well as the ^{15}N and $^{13}\text{C}_\alpha$, resulting in a 3D spectrum. Since the amide nitrogen is coupled to the C_α of its residue ($\text{C}_{\alpha i}$) and that of the preceding residue ($\text{C}_{\alpha i-1}$), both these transfers occur and the signals for both C_α are visible in the spectrum [10, 11].

In the HN(CO)CA experiment we can visualize a single signal for each amino acid, corresponding to the $C_{\alpha i-1}$. In this particular experiment, the magnetization is passed from $^1\text{H}_\text{N}$ to ^{15}N and then to the ^{13}C from the carbonyl group of the peptide bond. From there, the magnetization is transferred to $^{13}\text{C}_\alpha$, and the chemical shift is evolved. The magnetization is then transferred back, via ^{13}CO (the carbon from the carbonyl group of the peptide bond), to ^{15}N and $^1\text{H}_\text{N}$ for detection (Figure 2.1B). The chemical shift is only evolved for the $^1\text{H}_\text{N}$, ^{15}N and $^{13}\text{C}_\alpha$, but not for the ^{13}CO . This results in a spectrum with similar results to the HNCA, being selective for the $C_{\alpha i-1}$ [12].

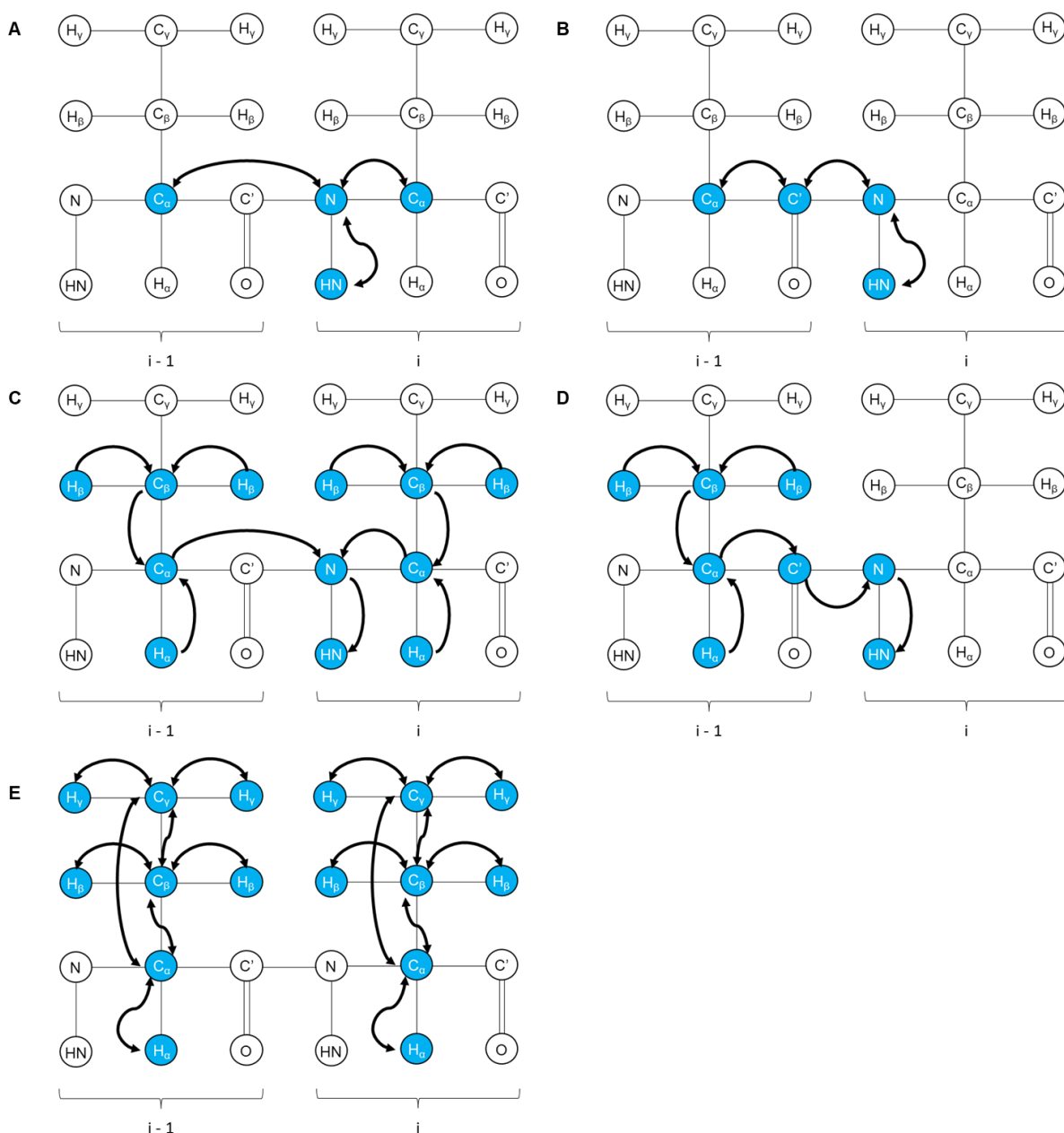


Figure 2.1 - Schemes representing magnetization transfer in 3D NMR experiments used in this Thesis. (A) HNCA; (B) HN(CO)CA; (C) HNCACB; (D) HN(CO)CACB; (E) TOCSY. Subscripts i and $(i-1)$ indicate a given and its previous amino acid, respectively. Adapted from [1, 13].

The HNCACB experiment is less sensitive (low signal-to-noise ratio) and is generally used as a complementary way to verify the signals, especially the C_β from each 2D $^1H, ^{15}N$ -HSQC signal. On a HNCACB experiment, the magnetization is transferred from $^1H_\alpha$ and $^1H_\beta$ to $^{13}C_\alpha$ and $^{13}C_\beta$, respectively, and then from $^{13}C_\beta$ to $^{13}C_\alpha$. From here it is transferred first to ^{15}N and then to ^1HN for detection. The transfer from $C_{\alpha i-1}$ can occur both to $^{15}N_{i-1}$ and $^{15}N_i$ or, viewed the other way, magnetization is transferred to $^{15}N_i$ from both $^{13}C_{\alpha i}$ and $^{13}C_{\alpha i-1}$ (Figure 2.1C). Thus, for each NH group there are two C_α and C_β peaks visible. The chemical shift is evolved simultaneously on $^{13}C_\alpha$ and $^{13}C_\beta$, therefore these appear in one dimension. The chemical shifts evolved in the other two dimensions are ^{15}N and ^1HN [14].

In the HN(CO)CACB experiment, both ^{13}C from the previous amino acid are visualized ($C_{\alpha i-1}$ and $C_{\beta i-1}$). This experiment also has a low signal-to-noise ratio. In this particular experiment (Figure 2.1D), the magnetization is transferred from $^1H_\alpha$ and $^1H_\beta$ to $^{13}C_\alpha$ and $^{13}C_\beta$, respectively, and then from $^{13}C_\beta$ to $^{13}C_\alpha$. From here it is transferred, at first, to ^{13}CO , then to ^{15}N and, finally, to ^1H for detection. The chemical shift is evolved simultaneously on $^{13}C_\alpha$ and $^{13}C_\beta$, therefore both appear in one dimension. The chemical shifts evolved in the other two dimensions are ^{15}N and ^1H . The chemical shift is not evolved on ^{13}CO [15].

In order to assign the protein's side chain, 3D TOCSY and 3D NOESY spectra are required.

The 3D TOCSY are constituted by 2D ^1H -TOCSY which peaks are dispersed in a third dimension, which is in this case the ^{13}C chemical shifts of the side chains (3D HCC(H)-TOCSY). It provides connectivities of the aliphatic side chains of individual amino acid residues, since the magnetization is transferred from the side-chain hydrogen nuclei to their attached ^{13}C nuclei connected by a chain of couplings. Finally, magnetization is transferred back to ^1H for detection (Figure 2.1E). This spectrum yields strips at each carbon frequency in the side chain in which all side-chain hydrogen resonances are visible [16]. The 3D NOESY follows the same principle, but it considers spatial interactions [3].

2.3 Thermodynamic model

In monoheme cytochromes, the redox potential values can be obtained directly from the Nernst equation and the E_{app} (*i.e.* the point at which the oxidized and reduced fractions of the protein are equal) corresponds to the reduction potential of the heme, and only the reduced and oxidized states may co-exist in solution. On the other hand, in the case of multiheme cytochromes, in addition to the reduced and oxidized states there also intermediate oxidation stages. Therefore, the E_{app} value determined for a multiheme cytochrome does not correspond to an individual reduction potential value of a particular heme. Instead, it describes the so-called macroscopic reduction potential and simply describes the overall redox behavior of that multiheme cytochrome. Potentiometric redox titrations or voltammetry measurements do not discriminate the individual redox potential of the individual hemes, which can only be obtain

in particular cases in which the reduction potentials of the hemes are considerably different, which is not often the case.

Particularly, in triheme cytochromes, three one-electron reversible transfer steps convert the fully reduced state (Stage 0, S_0) in the fully oxidized state (Stage 3, S_3) that define four different redox stages (Figure 2.2). In addition, in each stage, the protonable center(s) in the protein that modulate the hemes' reduction potential (redox-Bohr effect) may be protonated or deprotonated, which further increases the number of microstates in solution, yielding to 16 possible microstates (Figure 2.2). Also, the reduction potential of each heme is most often modulated by the oxidation stage of its neighbor (redox interactions) and by the solution pH (redox-Bohr interactions) due to the close spatial disposition of the heme groups within the protein. The magnitude of this effect is determined by the so-called redox-Bohr interactions (Figure 2.3), which measure the effect of the protonation state of the redox-Bohr center on the heme redox potentials. The redox-Bohr center is a protonable center, located in the vicinity of the hemes [17-20]. Taking as reference the fully reduced and protonated microstate, the energy of the other microstates can be described in the full range of pH and solution potential as sums of 10 parameters: the three energies of oxidation of the hemes (reduction potential values), the pK_a of the redox-Bohr center, the three interaction energies between each pair of hemes (redox interactions) and the three interaction energies between each heme and the redox-Bohr center (redox-Bohr interactions). When the interconversion between microstates within the same oxidation stage (intramolecular electron exchange) is fast on the NMR timescale and the interconversion between microstates of different oxidation stage (intermolecular electron exchange) is slow, the heme substituents will have different chemical shifts in each oxidation stage. Therefore, it is possible to follow the chemical shift variation of any heme substituent from its position in the reduced state to the fully oxidized state. Typically because of the regions they appear in the spectra and because of their three-proton intensity the heme methyl signals are the more appropriate choice to monitor and determine the heme oxidation fractions in 2D ^1H -EXSY NMR [21].

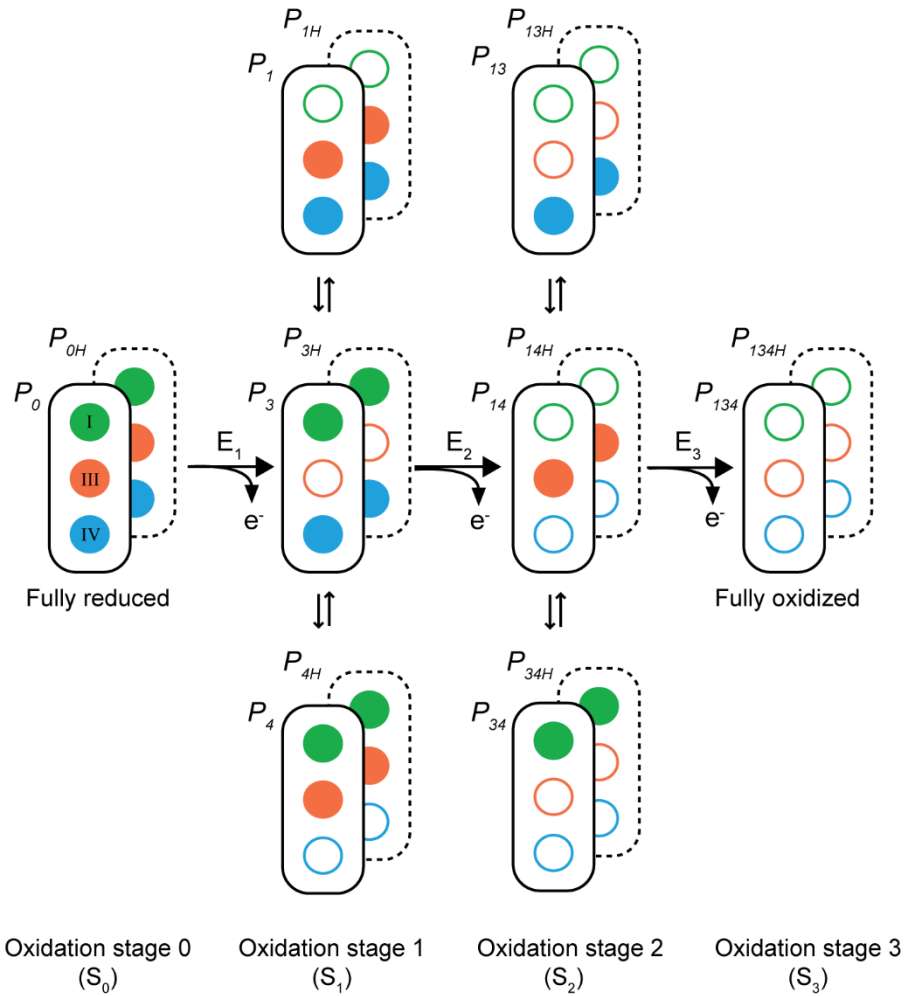


Figure 2.2 - Schematic representation of the electronic distribution for a triheme cytochrome with one redox-Bohr center, showing the 16 possible microstates. The filled and dashed rectangles represent the deprotonated and protonated microstates, respectively. The circles in each microstate represent the heme groups (green, orange, and blue for hemes I, III and IV, respectively), which can be either reduced (filled circles) or oxidized (open circles). The microstates are grouped according to the number of oxidized hemes, in four oxidation stages connected by three one-electron redox steps. P_{0H} and P_0 represent the reduced protonated and deprotonated microstates, respectively. P_{ijkH} and P_{ijk} indicate, respectively, the protonated and deprotonated microstates with heme(s) i , j , and k oxidized. E_1 , E_2 , and E_3 are the macroscopic reduction potentials for the first, second, and third oxidation steps, respectively.

The energy of each microstate relative to the reference microstate (fully reduced and protonated, P_{0H}) is then given by a simple sum of the appropriate energy terms amongst the four independent centers, the six possible two-center interactions and one term that accounts for the effect of the solution potential (SFE) in the oxidation stage S (Equation 6), and another for the proton chemical potential ($2.3RTpH$) added for the deprotonated forms (Equation 7):

$$G_{iH} = \sum g_i + \sum g_{ij} - SFE \quad (6)$$

$$G_i = G_{iH} + g_H + \sum g_{iH} - 2.3RTpH \quad (7)$$

In these equations, iH designates a particular protonated microstate with oxidized heme(s) group(s) i ($i = 1-3$); g_i the energy of oxidation of heme i ; g_{ij} the interaction energy between each pair of hemes i and j ; g_H the deprotonation energy of the fully reduced protein; g_{iH} the energy of interaction between the hemes and the redox-Bohr center; S the oxidation stage (that corresponds to the number of oxidized hemes); F the Faraday constant; E the redox potential of the solution; R the molar gas constant and T the absolute temperature.

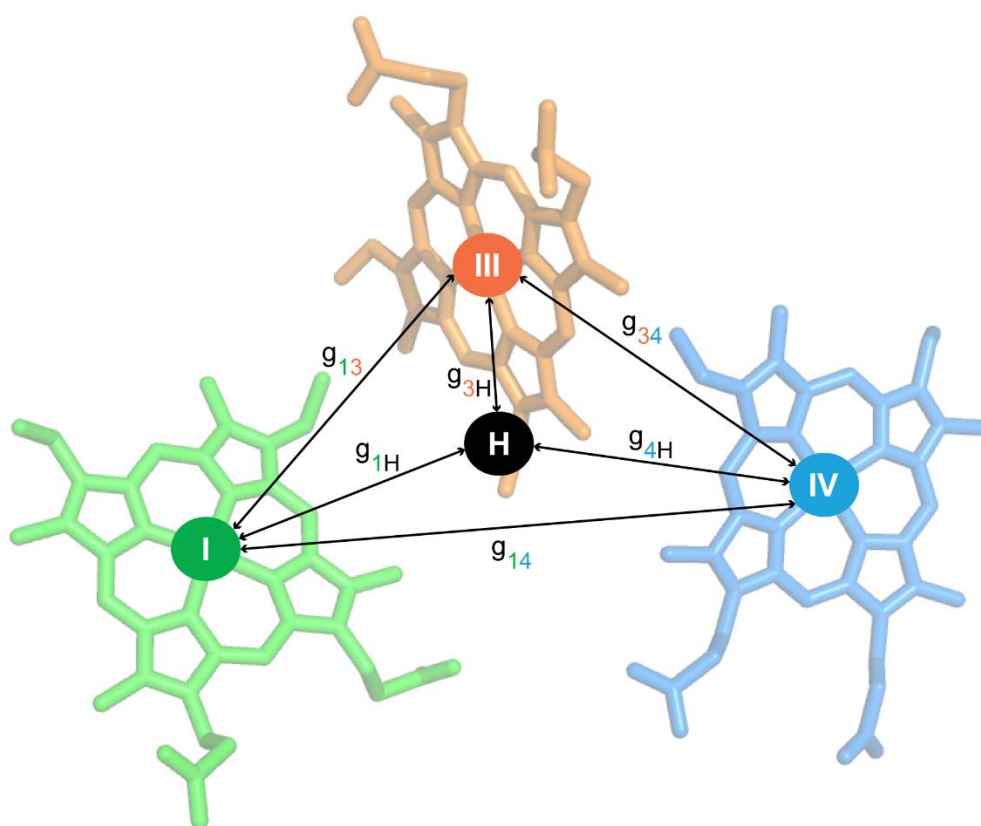


Figure 2.3 – Schematic representation of energy interactions and the interaction network of a multiheme cytochrome with three hemes (numbered I (green), III (orange) and IV (blue)) and one redox-Bohr center (black circle identified with an H). The terms g_{ij} and g_{iH} represent the interaction energies between the hemes (ij) and between the hemes and the redox-Bohr center (H), respectively. The individual heme oxidation energies are represented as g_1 , g_3 and g_4 , for hemes I, III and IV, respectively.

The referred energy values can be converted to reduction potential values, which further facilitates data interpretation, using the Nernst equation (Equation 8):

$$\Delta G = -nF\Delta E \quad (8)$$

Lastly, the fractional contribution of each microstate (P_i) can be determined by the Boltzmann equation (Equation 9):

$$P_i = e^{-G_i/RT} \quad (9)$$

Hence, the detailed thermodynamic characterization of multiheme cytochromes involves the determination of the individual heme reduction potentials, the redox interactions and the pK_a value(s) of the redox-Bohr center(s). To calculate these thermodynamic parameters, Turner and co-workers have developed a strategy that permits the determination of these parameters by combining the information from UV-visible redox titrations with NMR to determine the individual heme oxidation profiles [22]. An example of the application of this model is provided in Chapter 4.

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| 3.

**OPTIMIZATION OF THE PRODUCTION OF THE
PERIPLASMIC CYTOCHROMES P_{pcB}, P_{pcC} AND
P_{pcE} FROM *G. metallireducens* AND PRELIMINARY
BIOCHEMICAL CHARACTERIZATION**

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3. Optimization of the production of the periplasmic cytochromes PpcB, PpcC and PpcE from *G. metallireducens* and preliminary biochemical characterization

3.1 Introduction

As mentioned, the PpcA-family in *G. sulfurreducens* is composed by five triheme cytochromes – PpcA, PpcB, PpcC, PpcD and PpcE [1-3]. The members of this family have been biochemically characterized in detail with exception of PpcC [2]. In this particular case, the protein shows different conformations in solution at intermediate levels of oxidation that cause extremely broadening of the signals and, therefore impaired the determination. The correspondent family in *G. metallireducens*, PpcA, PpcB, PpcC, PpcE and PpcF is yet in the early stages of characterization. In fact, only PpcA and PpcF have been studied. However, while PpcA was characterized in detailed [4, 5], PpcF was only partially [6]. The detailed characterization of PpcF will be presented in the Chapter 4 of this Thesis. In addition to this, the characterization of the remaining three triheme cytochromes from *G. metallireducens* was also pursuit in this Thesis. Previously, the expression of cytochromes PpcC and PpcE from *G. metallireducens* were carried out with the same protocol as for PpcA from *G. sulfurreducens* [7]. However, the yields were very low, 0.05 (PpcC) and 0.03 (PpcE) mg/L of cell culture. Regarding cytochrome PpcB from *G. metallireducens*, no attempts were made and the gene encoding this protein was for the first time cloned in this Thesis..

In the present Chapter the cloning of PpcB gene is described, as well as the attempts to further optimize the expression of cytochromes PpcC and PpcE.

3.2 Materials and Methods

3.2.1 Expression tests – *E. coli* strains and optimal OD_{600nm} for induction

The expression of PpcC and PpcE was tested in different *E. coli* strains: BL21 (DE3), Tuner, JCB7123, JM109, C43 and SF110 all harboring the plasmid pEC86 containing the cytochrome *c* maturation gene cluster *ccmABCDEFGH* [8] and a chloramphenicol selection marker. In addition, all these strains were also transformed with the plasmid pVA203 containing either the gene encoding for cytochrome PpcC or PpcE, an ampicillin resistance gene, a lac promoter and an OmpA leader sequence [3, 9].

Transformed cells were first grown overnight at 30 °C in 5 mL 2xYT medium supplemented with 34 µg/mL chloramphenicol (CLO) and 100 µg/mL ampicillin (AMP), both from NZYTech, and then transferred to 50 mL of 2xYT medium supplemented with the same antibiotics until they reach an OD_{600nm} of either 0.7 or 1.5, at the same temperature. Then, protein expression was induced with 10 µM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultures were left at 30 °C overnight.

Aliquots were collected throughout the cell growth process to be later evaluated by 15% acrylamide/bis-acrylamide SDS-PAGE stained either for hemes with tetramethylbenzidine (TMBZ) or with BlueSafe (NZYTech) with the protein marker Precision Plus Protein™ Dual Xtra Standards (BioRad) ranging between 2 and 250 kDa. TMBZ is used to detect heme proteins. In this process TMBZ is used as a chromogenic electron donor and H₂O₂ is an alternative electron acceptor, which gives rise to light blue bands [10]. BlueSafe is a protein stain which consists of a safer alternative to the traditional Coomassie blue staining for detecting proteins in SDS-PAGE, which detects low concentration proteins (60 ng) within 10-15 minutes and down to 10 ng within 30-60 minutes.

After the selection of the *E. coli* strain and optimal OD_{600nm} for induction, 4 L of cell cultures were grown.

Following the cell harvesting, both cytochromes were purified through two chromatographic steps - a cation exchange chromatography followed by a molecular exclusion chromatography. Regarding PpcE, cation exchange chromatography was performed either on a XK 16/20 (GE Healthcare) column packed with UNOsphere S Ion Exchange (Bio-Rad) or on 2x5 mL BioScale MiniUnosphere™ S Cartridge (Bio-Rad) equilibrated with 10 mM Tris-HCl pH 8 and protein was eluted either with a 0-300 mM or 0-1 M NaCl gradient at a flow rate of 1 mL/min. In the case of PpcC, cationic columns either 2x5 mL HiTrap SP HP cartridges (GE Healthcare) or 2x5 mL BioScale MiniUnosphere™ S Cartridge (Bio-Rad) were equilibrated with 10 mM Tris-HCl pH 7 and protein was eluted with a 0-300 mM sodium chloride gradient at a flow rate of 1 mL/min. The obtained fractions were evaluated by 15% acrylamide/bis-acrylamide SDS-PAGE stained with

BlueSafe (NZYTech) and concentrated to 1 mL in Amicon Ultra centrifugal filter units (Ultra-4, MWCO 3 kDa) and equilibrated with 100 mM sodium phosphate buffer pH 8. Concentrated protein was loaded into a XK 16/70 Superdex 75 molecular exclusion column equilibrated with the same buffer. All chromatographic steps were performed on an ÄKTA Pure system (GE Healthcare). Samples purity was evaluated by 15% acrylamide/bis-acrylamide SDS-PAGE stained with BlueSafe and using the same protein marker as previously mentioned.

To determine the obtained protein yield, UV-visible absorption spectra in the oxidized and reduced states were acquired at room temperature on a UV-visible scanning spectrophotometer Ultraspec 2100pro (Amersham Biosciences, Switzerland) using quartz cuvettes with 1 cm path length. Protein reduction was achieved by adding crystals of sodium dithionite (Thermo Fisher Scientific) to the sample. Protein quantification was estimated using the molar extinction coefficient for reduced PpcA from *G. metallireducens* at 552 nm ($\epsilon_{552\text{nm}} = 118 \text{ mM}^{-1}\text{cm}^{-1}$) [5].

3.2.2 Expression tests – plasmids

Initially, cytochromes PpcC and PpcE were cloned and tested on pVA203 plasmid as cytochromes PpcB-E from *G. sulfurreducens* and PpcA and PpcF from *G. metallireducens*. However, the plasmid pCK32 were highly efficient in expressing PpcA from *G. sulfurreducens* [11]. While plasmid pCK32 is a pUC derivative containing the lac promoter and the OmpA leader sequence [11], pVA203, although derived from pCK32, has a NotI restriction site at the end of the leader peptide coding sequence and thus allow cloning without adding extra residues at the N-terminus of targets [6]. Also, pVA203 vector encodes for Skp, a periplasmic chaperone known to facilitate the folding of various proteins in the periplasm which was cloned immediately after the ampicillin-resistance gene to be expressed constitutively [9, 12, 13]. However, for not yet understandable reasons, expression yields vary with these two plasmid and cytochromes PpcC and PpcE were also cloned in pCK32 plasmid. The cytochrome PpcB, which was never cloned before, was cloned in both plasmids.

The desired sequences were inserted in the plasmids using Restriction-Free cloning method (RF cloning) [14]. RF cloning allows the insertion of any DNA fragment into a desired location within a circular plasmid, independent of restriction sites, ligation, or alterations in either the vector or the gene of interest, whilst the classical DNA cloning cleaving a destination plasmid and a target insert sequence with restriction enzymes, and then stitching them together with DNA ligase [15]. This technique starts with the design of two primers whose sequence are complementary both with the desired insert and the destination plasmid. The insert sequence is amplified with high-fidelity Polymerase Chain Reaction (PCR) and the resulting product is purified and used in a secondary PCR reaction,

as a “mega-primer”, where the destination plasmid is fully amplified. Then, the newly synthesized strands anneal and the “mega-primers” act as long complementary overhangs that circularize the plasmid, forming a nicked hybrid molecule. Lastly, the methylated parental plasmid is degraded by DpnI whilst the unmethylated in vitro synthesized hybrid plasmids remain untouched and prepared to transform competent bacteria (Figure 3.1). The primers used in the procedure were produced by Invitrogen and are presented in Table 3.1.

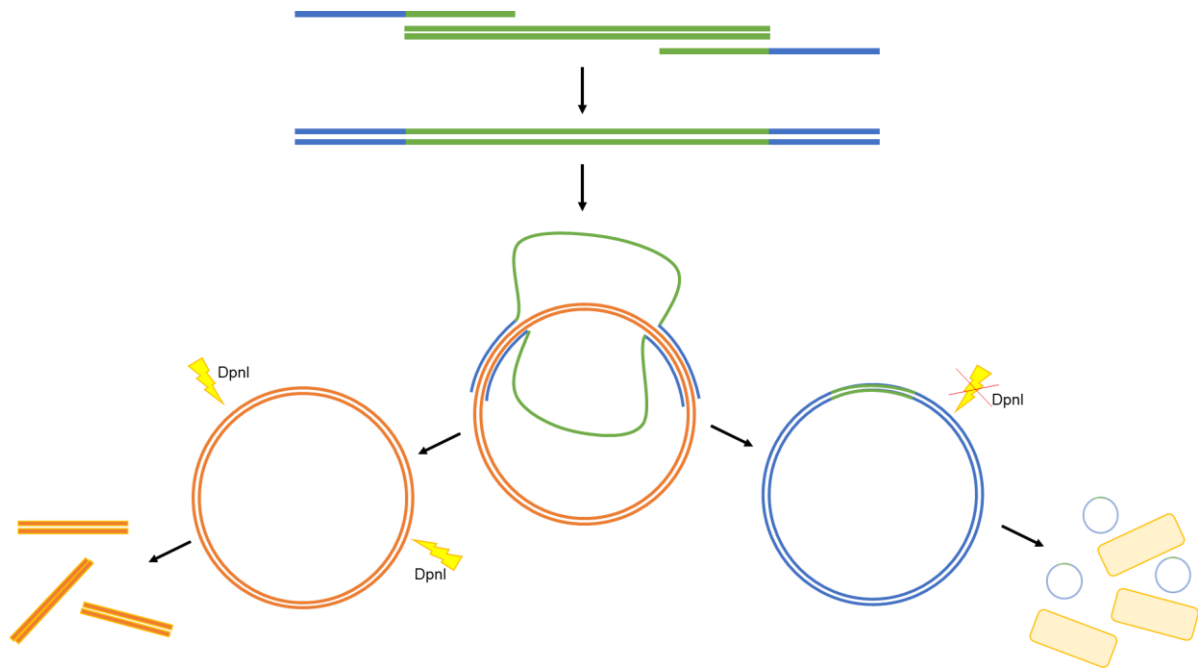


Figure 3.1 – Schematic representation of RF-cloning technique. Blue fragments represent the complementary sequence to the destination plasmid (orange). Green fragments represent the insert sequence. Adapted from [14].

Table 3.1 - Sequence of the DNA primers used to clone cytochrome PpcB in pCK32 and pVA203 plasmids (PpcB_pCK32 and PpcB_pVA203, respectively) and to clone cytochromes PpcC and PpcE in pCK32 plasmid (PpcC_pCK32 and PpcE_pCK32, respectively). The melting temperatures (T_m) for each primer and the annealing temperatures were calculated from the Thermo Scientific web tool (<https://www.thermofisher.com>). The sequences complementary to plasmid, either pCK32 or pVA203 are represented in blue and the insert sequence are represented in green and were designed using restricting free cloning website (<https://www.rf-cloning.org>).

	DNA sequence	T_m (°C)	Annealing Temperature (°C)
PpcB_pCK32 forward	5' TTTCGCTACCGTTGCTCAGGCAG CCGACACCCTCACCTTC 3'	81.7	65.3
PpcB_pCK32 reverse	5' AGCTTGTCGACGGAGCTCGAAT TTACTTCTTGTGGCACTCACCGC 3'	80.3	
PpcB_pVA203 forward	5' CTACCGTTGCGGCCCGCCG ACACCCTCACCTTC 3'	83.7	65.3
PpcB_pVA203 reverse	5' AGCTTGTCGACGGAGCTCGAATT TACTTCTTGTGGCACTCACCGC 3'	80.3	
PpcC_pCK32 forward	5' TTTCGCTACCGTTGCTCAGGCAAT CGAGACCATTACCTTTCCG 3'	78.8	62.7
PpcC_pCK32 reverse	5' AGCTTGTCGACGGAGCTCGA ATTAGCCACCTCCGTGACA 3'	72.0	
PpcE_pCK32 forward	5' TTTCGCTACCGTTGCTCAGGCAGC GGATACCATGATATCCCG 3'	79.7	62.2
PpcE_pCK32 reverse	5' AGCTTGTCGACGGAGCTCGAATCT ACTTCCTGTGGCAGAGG 3'	79.5	

The PCR samples were prepared with the conditions presented in Table 3.2 and the PCR cycling conditions used are indicated in Table 3.3. Negative controls, without template DNA, were also performed.

Table 3.2 - Composition of the final PCR mix. The Phusion DNA polymerase, Phusion buffer and nucleotides are from Thermo Fisher Scientific. 1 U is the amount of enzyme needed to convert in one minute 1 μ mole of substrate.

PCR reaction mix components	Final concentration
Phusion High Fidelity Buffer	1x
Nucleotides	200 μ M
Forward Primer	0.5 μ M
Reverse Primer	0.5 μ M
Template DNA	0.5 ng/ μ L
Phusion DNA Polymerase	0.02 U/ μ L

Table 3.3 - Cycling conditions of the first PCR. The annealing/extension time was calculated according to the manufacturer's indication of 15-30 seconds/kb. *indicates the different annealing temperatures that were used: 65 °C for PpcB_pCK32 and PpcB_pVA203; and 63 °C for PpcC_pCK32 and PpcE_pCK32.

PCR step	Temperature (°C)	Time (seconds)	Cycles
Initial denaturation	98	30	1
Denaturation	98	8	34
Annealing	65/63*	20	
Extension	72	10	
Final extension	72	600	1
Final hold	16	-	-

The size of the PCR products was confirmed by 1.5% agarose gel using GeneRuler 1kb DNA ladder (New England BioLabs) and purified using the NZYGelpure (NZYTech) and quantified with NanoDrop™ (Thermo Fisher Scientific). Then, a second PCR was carried out to insert the obtained PCR products ("mega-primer") in the plasmid of interest. The conditions for the prepared PCR samples are the same as in Table 3.2 and cycling conditions are presented in Table 3.4.

Table 3.4 - Cycling conditions of the second PCR. The annealing/extension time was calculated according to the manufacturer's indication of 15-30 seconds/kb.

PCR step	Temperature	Time (seconds)	Cycles
Initial denaturation	98	30	1
Denaturation	98	8	20
Annealing	69	20	
Extension	72	100	
Final extension	72	600	1
Final hold	16	-	-

PCR products were digested with 1U (the amount of DpnI required to digest completely 1 µg of plasmid DNA in 50 µL of the reaction mixture at 37 °C for one hour) of DpnI (NZYTech) for 3h at 37 °C, which is the optimal activity temperature of the enzyme. Then, DpnI was inactivated at 80 °C for 20 minutes.

E. coli DH5α competent cells were transformed with the plasmids using the heat shock method [16] which consist in incubate the cells on ice for 30 minutes, followed by 1-minute incubation at 42 °C and 1-minute incubation on ice again. Then, 500 µL of Luria Bertrani (LB) medium was added and cells were grown for 1 h at 37 °C, 200 rpm. Next, transformed cells were plated in solid LB medium supplemented with 100 µg/mL of AMP and incubated overnight at 37 °C. Positive and negative controls were always carried out. Selected colonies

were inoculated into 5 mL of LB medium supplemented with 100 µg/mL of AMP and grown overnight at 37 °C, 200 rpm. The plasmid was isolated from the grown culture using the NZYMiniprep kit (NZYTech) according to the manufacturer's instructions and the obtained plasmid was quantified using the NanoDrop™. The sequence of the obtained DNA plasmids was confirmed by DNA sequencing performed by STAB VIDA. Then, the new plasmids were used to express the respective cytochromes and expression tests were performed as in section 3.2.1 to select the best experimental conditions.

3.2.3 NMR studies

3.2.3.1 Sample preparation

Purified cytochromes PpcC and PpcE were lyophilized and prepared in pure ²H₂O (CIL isotopes) 80mM phosphate buffer pH 8, with NaCl (final ionic strength 250mM) to a final concentration of 0.8 M. The fully reduced sample was achieved by sealing the NMR tube with a gas-tight serum cap and by flushing the air out from the sample, to avoid possible oxidation. Then, gaseous hydrogen was added in the presence of catalytic amounts of Fe-hydrogenase isolated from *Desulfovibrio vulgaris* (Hildenborough). Fully reduction of the samples was confirmed by 1D ¹H NMR.

Samples for NMR redox titration was prepared with approximately 80 µM concentration, in 80 mM phosphate buffer pH 8 prepared in pure ²H₂O, with NaCl (250 mM of final ionic strength). Partially oxidized samples were obtained by firstly removing the hydrogen with nitrogen and then by adding controlled amounts of air into the NMR tube with a Hamilton syringe through the gas-tight serum cap.

3.2.3.2 NMR experiments

All NMR experiments were acquired in a Bruker Avance III 600 MHz spectrometer equipped with a triple-resonance cryoprobe (TCI). The ¹H chemical shifts are referenced to DSS at 0 ppm by using the residual water signal as an internal secondary reference, as previously described [17]. All the spectra were processed using TopSpin (Bruker BioSpin, Karlsruhe, Germany).

3.2.3.2.1 Experiments in the fully reduced state

For the assignment of the heme methyl substituents of PpcC and PpcE in the fully reduced state, 2D ¹H-NOESY and 2D ¹H-TOCSY experiments were acquired at 15 °C. The 2D ¹H-NOESY spectrum was acquired with a mixing time of 80 ms, collecting 4096 (t₂) x 512 (t₁) data points and spectral width of 90.1 kHz, with 136 scans per increment. The 2D ¹H-TOCSY spectrum was acquired with a mixing time of 60 ms and collecting 2048 (t₂) x 512 (t₁) data points, with the same spectral width and number of scans. The 1D ¹H NMR spectrum was

acquired at 15 °C, with water pre-saturation, collecting 32k data points to cover a spectral width of 42 kHz.

3.2.3.2.2 Experiments in the fully oxidized state

For the assignment of the heme methyl substituents of PpcE in the fully oxidized state 2D ^1H , ^{13}C -HMQC, 2D ^1H -NOESY and 2D ^1H -TOCSY experiments were acquired at 15 °C. The 2D ^1H , ^{13}C -HMQC spectrum was acquired by collecting 4096 (t_2) $\times$ 256 (t_1) data points to cover a sweep width of 26.3 kHz in the ^1H dimension and 52.8 kHz in the ^{13}C dimension, with 320 scans per increment. The 2D ^1H -NOESY spectrum was acquired with a mixing time of 80 ms, collecting 4096 (t_2) $\times$ 512 (t_1) data points, to cover a sweep width of 26.3 kHz, with 128 scans per increment. The 2D ^1H -TOCSY spectrum was acquired with a mixing time of 45 ms, collecting 2048 (t_2) $\times$ 512 (t_1) data points to cover a sweep width of 26.3 kHz, with 160 scans per increment. The 1D ^1H NMR spectrum was acquired at 15 °C, with water pre-saturation, collecting 32k data points to cover a spectral width of 42 kHz.

3.2.3.2.3 Experiments in intermediate stages of oxidation

To monitor the individual heme oxidation patterns of PpcE, a series of 2D ^1H -EXSY with the sample poised at different degrees of oxidation was acquired. All the 2D ^1H -EXSY spectra were acquired with a mixing time of 25 ms at 15 °C, collecting 2048 (t_2) $\times$ 256 (t_1) data points to cover a sweep width of 25.2 kHz, with 256 scans per increment. 1D ^1H NMR spectra, collecting 32k data points to cover a spectral width of 25.3 kHz, were obtained before and after each 2D NMR spectrum to check for any changes in the oxidation state of the sample during the experiments.

3.2.4 Potentiometric redox titrations

Potentiometric redox titrations of PpcE were followed by visible spectroscopy and performed inside an anaerobic glove box (MBraun) with oxygen levels kept under 1 ppm. The redox titrations were performed at 15 °C and the visible spectra were recorded in an Evolution 300 spectrophotometer (Thermo Scientific). Protein solutions (12 μM) were prepared in 80 mM sodium phosphate with NaCl (250 mM final ionic strength) at pH 7.0 and 8.0. The solution redox potential was measured using a combined Pt/Ag/AgCl electrode, calibrated with quinhydrone saturated solutions at pH 4 and pH 7. The following mixture of redox mediators was added to the solution to a final concentration of approximately 1.8 μM , as described in the literature [18], to ensure equilibrium between the redox center of the protein and the working electrode: galloxyanine ($E^0 = +21$ mV), methylene blue ($E^0 = +11$ mV), indigo tetrasulfonate ($E^0 = -30$ mV), indigo trisulfonate ($E^0 = -70$ mV), indigo disulfonate ($E^0 = -110$ mV), 2-hidroxy-1,4-naphthoquinone ($E^0 = -152$ mV), anthraquinone-

2,6-disulfonate ($E^{\circ} = -184$ mV), anthraquinone-2-sulfonate ($E^{\circ} = -225$ mV), safranine O ($E^{\circ} = -280$ mV), neutral red ($E^{\circ} = -325$ mV), benzyl viologen ($E^{\circ} = -345$ mV), diquat ($E^{\circ} = -350$ mV) and methyl viologen ($E^{\circ} = -440$ mV). A [protein]:[redox mediators] ratio of approximately 10 was found to be optimal to assure good redox equilibrium with the electrode and to avoid specific interactions of mediator molecules with the protein. To check for hysteresis and reproducibility, each redox titration was performed in both oxidative and reductive directions, using sodium dithionite as reducing agent and potassium ferricyanide as oxidizing agent. The experiments were performed at least two times and the reduction potentials (relative to the normal hydrogen electrode, NHE) were found to be reproducible within ± 5 mV. Each measured solution redox potential was corrected to the reference value by the addition of 207 mV. The reduced fraction of PpcE was determined by integrating the area of the α -peak (553 nm) above the line connecting the flanking isosbestic points (540 and 562 nm) to subtract the optical contribution of the redox mediators.

3.2.5 AlphaFold models

Cytochromes PpcB, PpcC and PpcE from *G. metallireducens* AlphaFold models were calculated with the Google Colab platform [19] through Chimera X 1.3 [20] which does not use structural templates (homologous structures) and searches for a smaller set of protein sequences compared with the full opensource AlphaFold [21]. Among the top five models calculated by default, only the best model was evaluated and presented. The quality of the models was evaluated based on the per-residue estimate of confidence produced by AlphaFold, pLDDT (predicted local distance difference test). PyMol was used to represent the final models [22].

3.3 Results and discussion

3.3.1 Expression and purification optimization

The first time PpcE cytochrome was expressed in our laboratory it followed the protocol used for PpcA from *G. sulfurreducens* [7]. The purification stage encompassed two chromatographic steps: a cation exchange chromatography in 2x5 mL BioScale MiniUnosphere™ S Cartridge followed by size exclusion chromatography (Figure 3.2). The final protein's yield was 0.03 mg/L per cell culture.

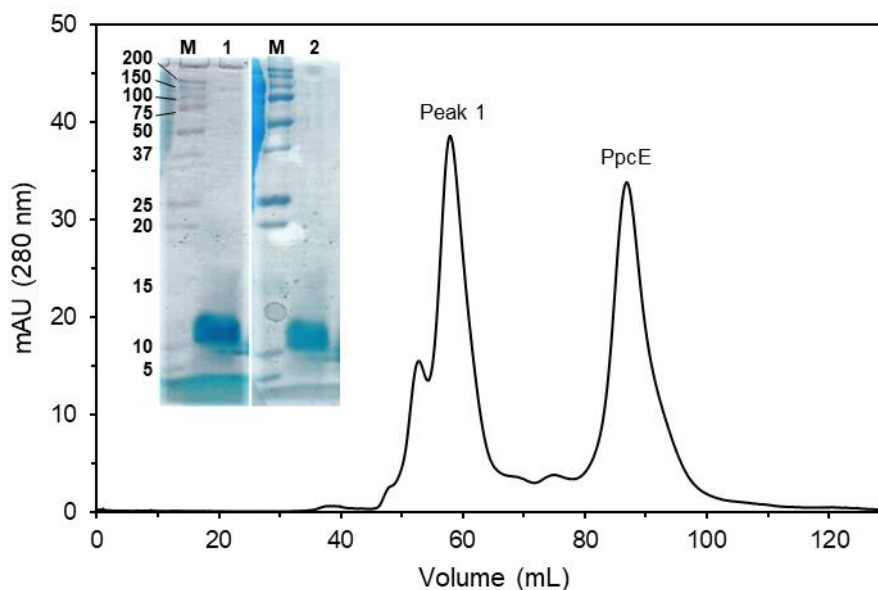


Figure 3.2 – Molecular exclusion chromatography of PpcE from *G. metallireducens* and SDS-PAGE of collected fractions. Lane M corresponds to the molecular weight marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lane 1 and 2 corresponds to the collected fractions of pure PpcE stained with either TMBZ or BlueSafe, respectively.

The low yield obtained is insufficient to assist a structural and functional characterization of the protein and hence further optimization was pursuit in this Thesis. Different experimental conditions both for the protein expression and purification stages were further explored. Protein's expression was tested in different bacterial strains and induction at either $OD_{600nm} \sim 0.7$ or $OD_{600nm} \sim 1.5$. Expression of PpcE was tested in *E. coli* strains BL21 (DE3), Tuner, JCB7123, JM109, C43 and SF110, harboring the pEC86 plasmid [23, 24]. After transformation with the plasmid containing the *ppcE* gene, the OD_{600nm} of each strain was monitored every two hours until it reached the values 0.7 and 1.5 (except for C43, JCB7123 and BL21(DE3) strains) at which expression was induced. SDS-PAGE gels were used to evaluate the different expression levels of each strain (Figure 3.3). Though all strains produced the protein, higher expression levels were obtained with strains JCB7123 with induction at $OD_{600nm} \sim 1.5$. For this reason, these conditions were chosen as starting point for the next optimization steps (see below).

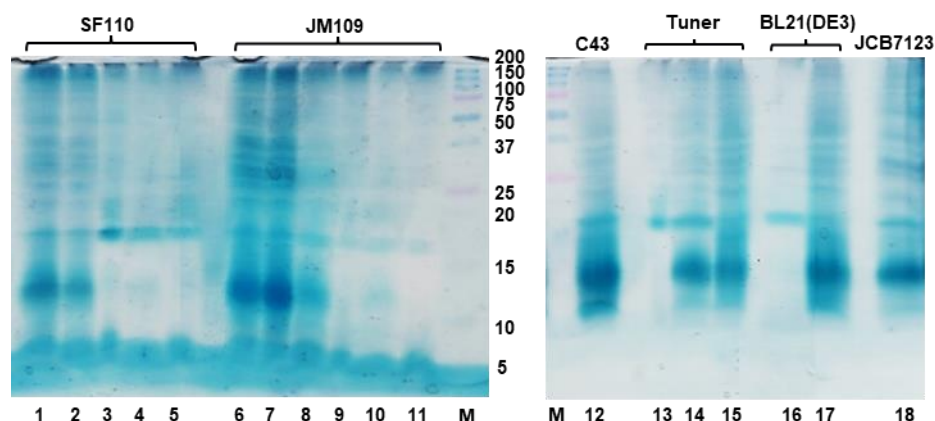


Figure 3.3 - SDS-PAGE stained with TMBZ of PpcE from *G. metallireducens* expressed in different *E. coli* strains and induced at either $OD_{600nm} \sim 0.7$ or $OD_{600nm} \sim 1.5$. M lanes correspond to the protein marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lanes 5, 11 and 16 correspond to aliquots of the sample before induction at $OD_{600nm} \sim 0.7$. Lanes 4 and 10 correspond to aliquots of the sample after 2h of induction at $OD_{600nm} \sim 0.7$. Lanes 3, 9 and 13 correspond to aliquots of the sample before induction at $OD_{600nm} \sim 1.5$. Lanes 8 and 14 correspond to aliquots of the sample after 2h of induction at $OD_{600nm} \sim 1.5$. Lanes 2, 7, 17 and 18 correspond to aliquots of the sample after overnight induction at $OD_{600nm} \sim 0.7$. Lanes 1, 6, and 15 correspond to aliquots of the sample after overnight induction at $OD_{600nm} \sim 1.5$. The strains to which each lane corresponds are identified at the top of the figure.

After expression of cytochrome PpcE at a larger scale in the selected conditions, its purification encompassed a cation exchange chromatography step using a XK 16/20 (GE Healthcare) column packed with UNOsphere S Ion Exchange (Bio-Rad) equilibrated with 10 mM Tris-HCl pH 8. As for all members of the PpcA-family from *G. sulfurreducens* and PpcA and PpcF from *G. metallireducens*, an elution gradient of 0-300 mM NaCl was used. However, this gradient was not sufficient to elute the protein and a new cation exchange chromatography step was carried out, this time on 2x5 mL BioScale MiniUnosphere™ S cartridge (Bio-Rad) equilibrated with 10 mM Tris-HCl pH 8 and an elution gradient of 0-1 M NaCl. The obtained chromatogram is indicated in Figure 3.4A. The fractions corresponding to cytochrome PpcE were then collected and loaded into a XK 16/70 Superdex 75 molecular exclusion column equilibrated with 100 mM sodium phosphate buffer pH 8. The obtained PpcE's fractions were analyzed by SDS-PAGE (Figure 3.4B). The obtained fraction was pure, and the yield obtained increased from 0.03 to 0.15 mg/L of cell culture.

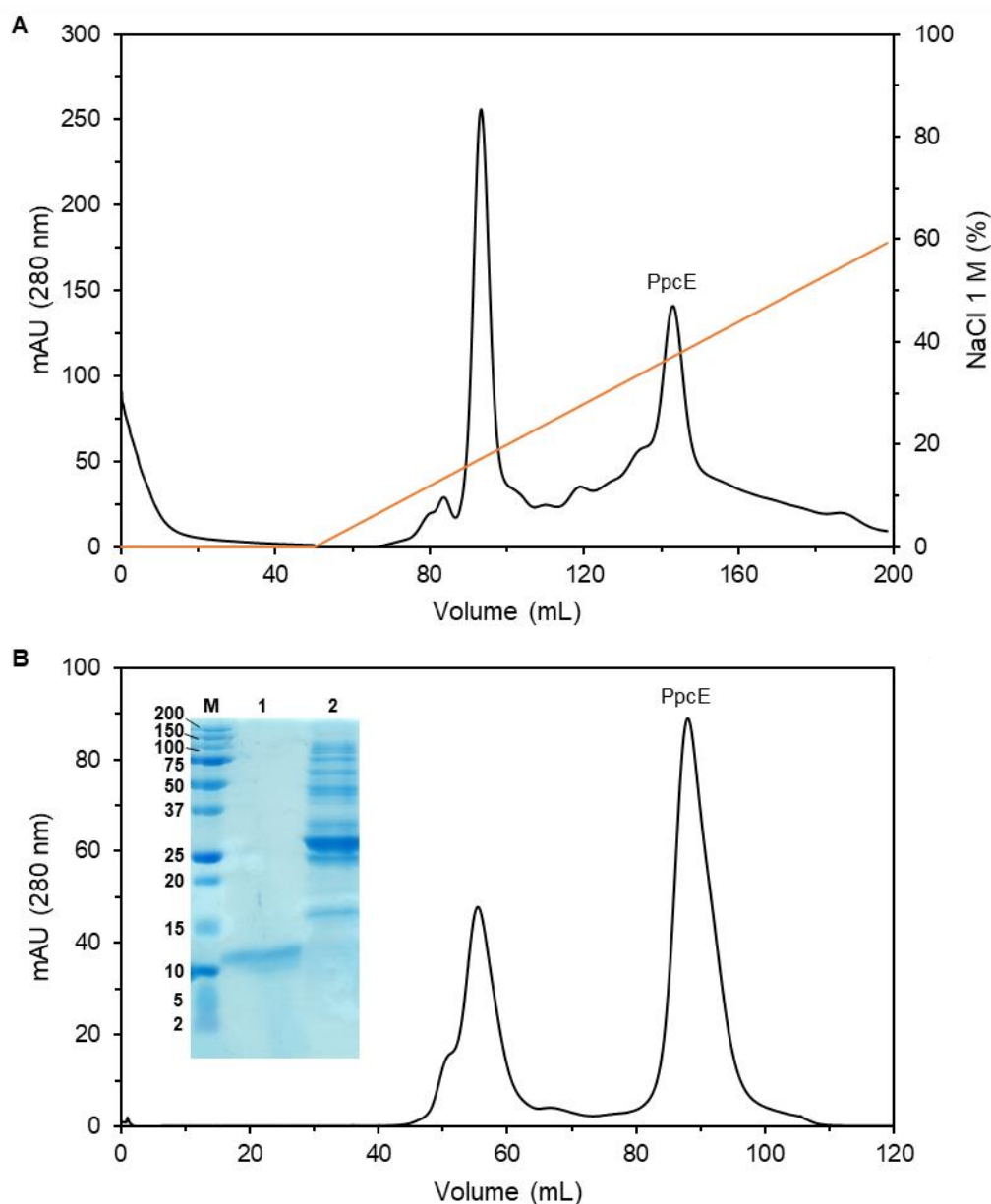


Figure 3.4 – Elution profiles of PpcE from *G. metallireducens* expressed in *E. coli* JCB7123 cells. (A) Cation exchange chromatography elution profile on 2x5 mL BioScale MiniUnosphere™ S cartridge. The primary and secondary y-axis report the variation of absorbance at 280 nm (black line) and the NaCl gradient profile (orange line), respectively. The peak corresponding to the PpcE fractions is identified. (B) Molecular exchange chromatography elution profile and SDS-PAGE of collected fractions. The peak corresponding to the PpcE fractions is identified. Lane M corresponds to the molecular weight marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lanes 1 and 2 correspond to the collected fractions of pure PpcE and the periplasmic fraction, respectively.

Regarding PpcC from *G. metallireducens*, the first time it was expressed it also followed the protocol for PpcA from *G. sulfurreducens* and the yield obtained was 0.05 mg/L of cell culture. Like PpcE, the expression of PpcC was firstly evaluated using different *E. coli* strains: BL21 (DE3), Tuner, JCB7123, JM109, C43 and SF110. Expression in all strains was monitored every two hours until it reached the values 0.7 and 1.5 at which expression was

induced. SDS-PAGE gels were used to evaluate the different expression levels of each strain (Figure 3.5). The data obtained, showed that the best results were obtained for *E. coli* BL21 (DE3) and JM109 strains with induction at $OD_{600nm} \sim 1.5$ and strain C43 with induction at $OD_{600nm} \sim 0.7$. However, BL21 (DE3) had already proven to lead to poor expression yields. In what concerns C43 and JM109, the latter showed the most reddish pellets and for that reason was selected for the next optimization steps (see below).

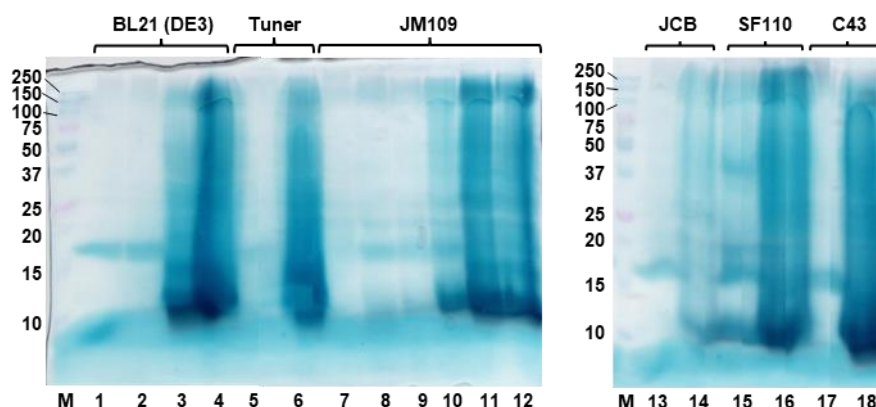


Figure 3.5 - SDS-PAGE stained with TMBZ of PpcC from *G. metallireducens* expressed in different *E. coli* strains and induced at either $OD_{600nm} \sim 0.7$ or $OD_{600nm} \sim 1.5$. M lanes correspond to the protein marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lanes 1, 5, 7, 13 and 17 correspond to aliquots of the sample before induction at $OD_{600nm} \sim 0.7$. Lane 9 corresponds to aliquots of the sample after 2h of induction at $OD_{600nm} \sim 0.7$. Lanes 2, 8 and 15 correspond to aliquots of the sample before induction at $OD_{600nm} \sim 1.5$. Lane 10 corresponds to aliquots of the sample after 2h of induction at $OD_{600nm} \sim 1.5$. Lanes 3, 6, 11, 14 and 18 correspond to aliquots of the sample after overnight induction at $OD_{600nm} \sim 0.7$. Lanes 4, 12, and 16 correspond to aliquots of the sample after overnight induction at $OD_{600nm} \sim 1.5$. The strains to which each lane corresponds are identified at the top of the figure.

Following the production of PpcC in the chosen conditions, its purification was firstly carried out with a 2x5 mL BioScale Mini UNOsphere™ S Cartridges. During the loading the flow-through showed a reddish color, suggesting that the protein did not fully bound to the column. Indeed, the profile of the obtained chromatogram (Figure 3.6A), as well as the color of the obtained fractions confirm that the protein did not elute in one peak, but rather along the entire ionic strength gradient. Thus, another cation exchange chromatography was performed, this time using 2x5 mL HiTrap SP HP cartridges (Figure 3.6B) and an elution gradient of 0-300 mM NaCl. In this case it was possible to obtain a single peak, and fractions corresponding to PpcC were loaded into the molecular exclusion column and analyzed by SDS-PAGE (Figure 3.6C). The yield obtained using this protocol increased from 0.05 to 0.54 mg/L of cell culture.

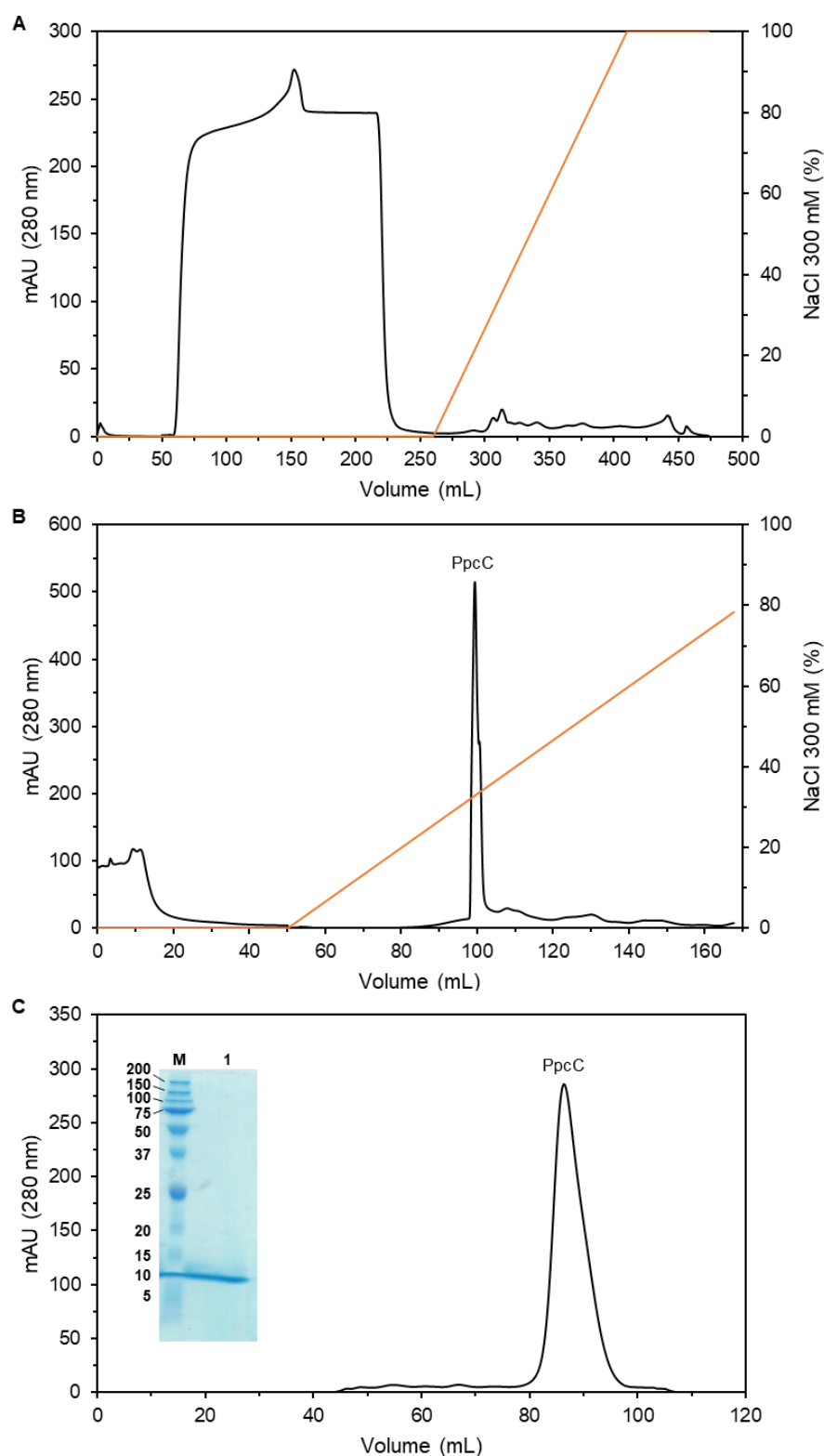


Figure 3.6 - Elution profiles of PpcC from *G. metallireducens* expressed in *E. coli* JM109 cells. (A) Cation exchange chromatography elution profile on 2x5 mL BioScale™ Mini UNOsphere S Cartridges. (B) Cation exchange chromatography elution profile on 2x5 mL HiTrap SP HP cartridges. (C) Molecular exchange chromatography elution profile and SDS-PAGE of collected fractions. Lane M corresponds to the molecular weight marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lane 1 corresponds to the collected fractions of pure PpcC. The peak corresponding to the PpcC fractions is identified. The primary and secondary y-axis report the variation of absorbance at 280 nm (black line) and the NaCl gradient profile (orange line), respectively. The peak corresponding to the PpcC fractions is identified.

Regarding PpcC and PpcE cloned in plasmid pCK32 instead of pVA203, their expression in *E. coli* was tested in strains BL21 (DE3) and JM109 or BL21 (DE3) and JCB7123 for cytochromes PpcC and PpcE, respectively, since BL21 (DE3) is the *E. coli* strain with better results in all PpcA-family members in *G. sulfurreducens* and PpcA and PpcF in *G. metallireducens*. Also, PpcC and PpcE cloned in pVA203 showed the best results with strains JM109 and JCB7123, respectively. SDS-PAGE was performed to analyze the results and aliquots of PpcC and PpcE cloned in pVA203 in *E. coli* strains JM109 and JCB7123, respectively, were loaded in the gel for comparison (Figure 3.7).

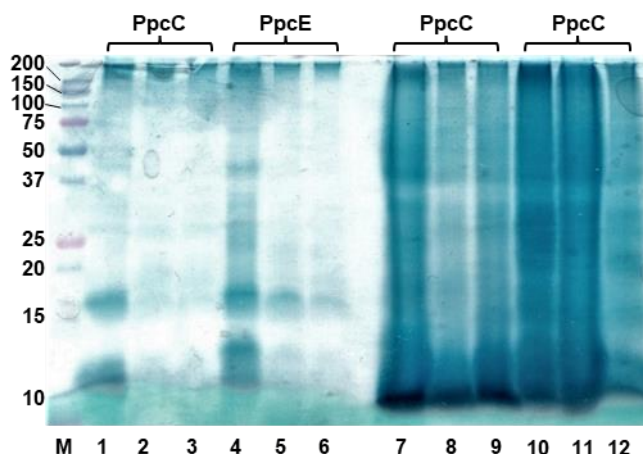


Figure 3.7 - SDS-PAGE stained with TMBZ of PpcC and PpcE from *G. metallireducens* expressed in different *E. coli* strains in both pVA203 and pCK32 plasmids. M lane correspond to the protein marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lanes 1, 2, 4, 5, 7, 8, 10 and 11 correspond to the cytochrome expressed in plasmid pCK32. Lanes 3, 6, 9 and 12 correspond to the cytochrome expressed in plasmid pVA203. Lanes 1 and 6 correspond to aliquots of the sample before induction at $OD_{600nm} \sim 1.5$. Lanes 7 to 12 correspond to aliquots of the sample after overnight induction at $OD_{600nm} \sim 1.5$. Lanes 1, 4, 7 and 10 correspond to expression in *E. coli* strain BL21 (DE3). Lanes 2, 3, 8 and 9 correspond to expression in *E. coli* strain JM109. Lanes 5, 6, 11 and 12 correspond to expression in *E. coli* strain JCB7123

Given the SDS-PAGE results, *E. coli* strains BL21 and JCB7123 for the cytochromes PpcC and PpcE, respectively, expressed with the plasmid pCK32, seem to have the highest expression levels. Hence, we proceed with the expression under these conditions, with induction at $OD_{600nm} \sim 1.5$, and purification steps were performed as previously optimized – cation exchange on 2x5 mL HiTrap SP HP cartridges using an elution gradient of 0-300 mM NaCl for cytochrome PpcC and on 2x5 mL BioScale MiniUnosphere™ S cartridges using an elution gradient of 0-1 M NaCl for cytochrome PpcE, followed by molecular exclusion chromatography on XK 16/70 Superdex 75. After the purification, cytochrome PpcC yield decreased substantially compared to the previous conditions, whereas for PpcE its expression yield increased from 0.15 to 0.63 mg/L of cell culture.

In what concerns cytochrome PpcB, its gene was cloned using the RF cloning technique. The resulting PCR products were transformed in *E. coli* DH5 α , grown in LB media and the plasmid was isolated from the grown culture using the NZYMiniprep kit, quantified using the NanoDrop™ and the DNA sequences were confirmed by DNA sequencing.

Cytochrome PpcB expression in both plasmids pCK32 and pVA203 was tested in *E. coli* strains BL21 (DE3), Tuner, JCB7123, JM109, C43 and SF110, with induction at $OD_{600nm} \sim 1.5$

and SDS-PAGE of the collected aliquots was performed to monitor the expression (Figure 3.8).

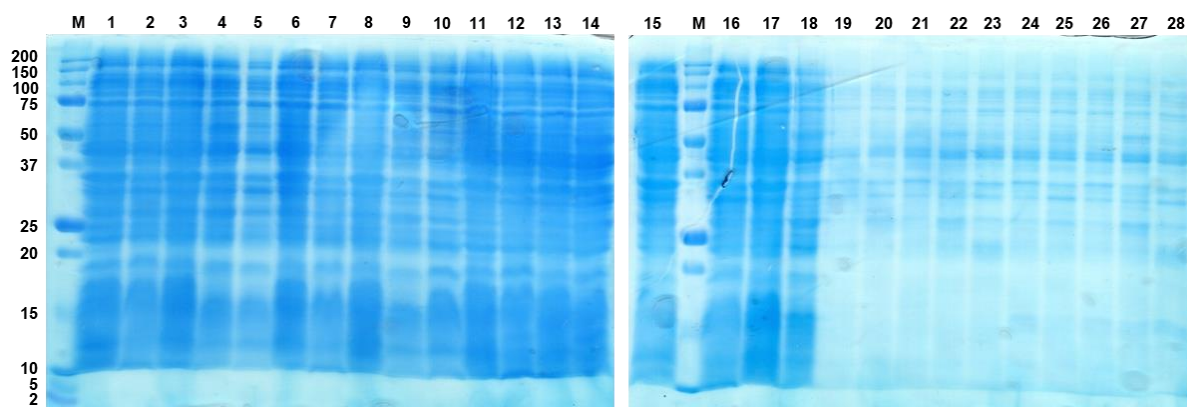


Figure 3.8 - SDS-PAGE stained with TMBZ of PpcB from *G. metallireducens* expressed in different *E. coli* strains in both pVA203 and pCK32 plasmids. M lanes correspond to the protein marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lanes 1 to 9 and 19 to 23 correspond to expression in plasmid pVA203. Lanes 10 to 18 and 24 to 28 correspond to expression in plasmid pCK32. Lanes 1, 2, 10, 11, 19 and 24 correspond to expression in *E. coli* strain BL21 (DE3). Lanes 3, 4, 12, 13, 20 and 25 correspond to expression in *E. coli* strain JM109. Lanes 5, 6, 14, 15, 21 and 26 correspond to expression in *E. coli* strain Tuner. Lanes 7, 8, 16, 17, 22 and 27 correspond to expression in *E. coli* strain SF110. Lanes 9, 18, 23 and 28 correspond to expression in *E. coli* strain JCB7123. Lanes 2, 4, 5, 7, 10, 12, 15 and 16 corresponds to aliquots of the sample after 2h of induction at $OD_{600nm} \sim 1.5$. Lanes 1, 3, 6, 8, 9, 11, 13, 14, 17 and 18 correspond to aliquots of the sample after overnight induction at $OD_{600nm} \sim 1.5$. Lanes 19 to 28 correspond to aliquots of the sample before induction at $OD_{600nm} \sim 1.5$.

The results showed that, the best condition was obtained with plasmid pVA203 and *E. coli* Tuner strain with expression induction at $OD_{600nm} \sim 1.5$. As for the other homologs, purification was performed with two chromatographic steps (Figure 3.9). The obtained fractions were analyzed by SDS-PAGE to evaluate the purity of the protein (see inset in Figure 3.9B). A yield of 3 mg/L of cell culture was obtained for PpcB.

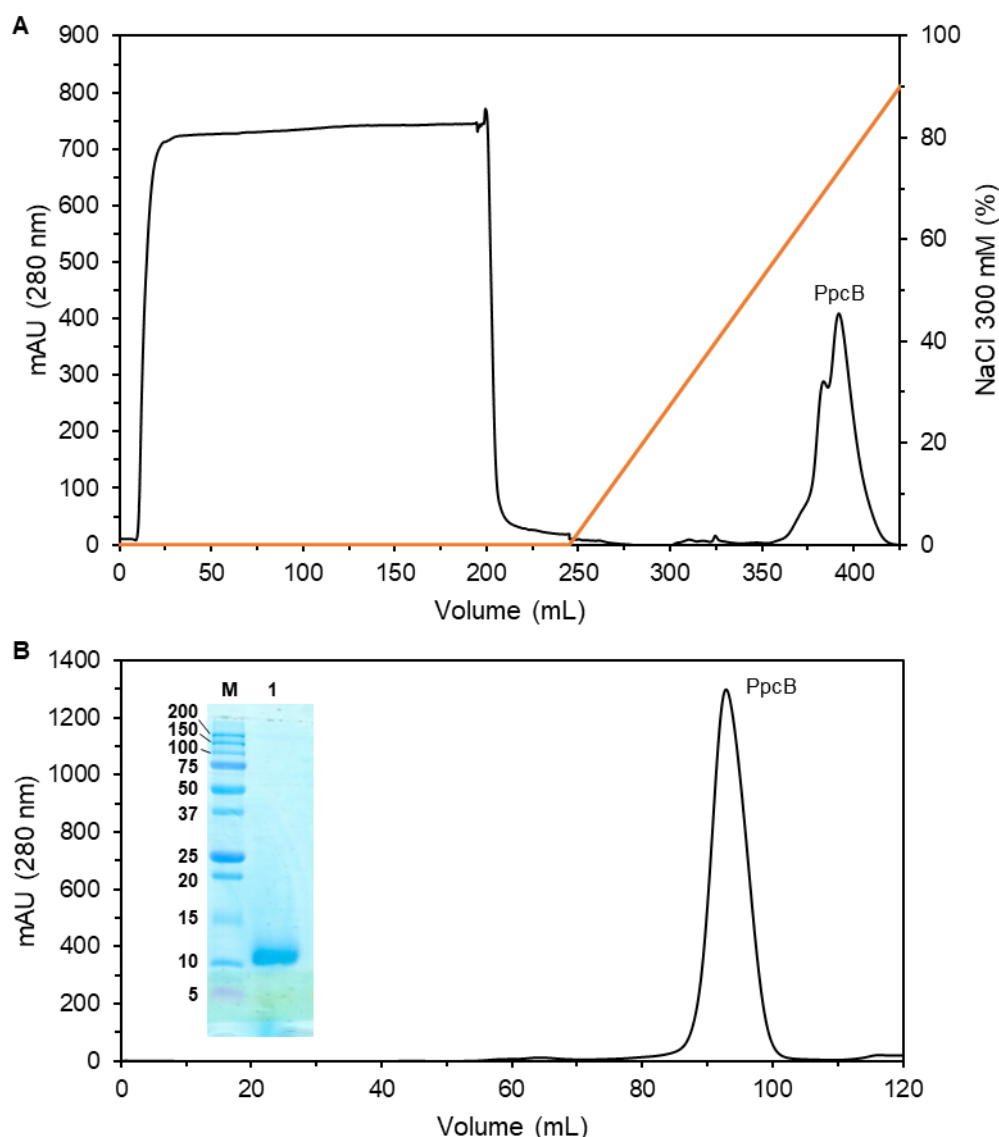


Figure 3.9 – Elution profiles of PpcB from *G. metallireducens* expressed in *E. coli* Tuner cells. (A) Cation exchange chromatography elution profile on 2x5 mL BioScale MiniUnosphere™ S cartridges. (B) Molecular exchange chromatography elution profile and SDS-PAGE of collected fractions. Lane M corresponds to the molecular weight marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lane 1 corresponds to the collected fractions of pure PpcB. The peak corresponding to the PpcB fractions is identified.

3.3.2 Determination of the spin state of the heme groups

The spin state of the hemes of cytochromes PpcB, PpcC and PpcE from *G. metallireducens* was investigated by UV-visible (Figure 3.10). The spectral features displayed by the cytochromes are typical of low-spin hexacoordinated hemes [5, 25]. The maxima absorbance of each cytochrome is presented in Table 3.5.

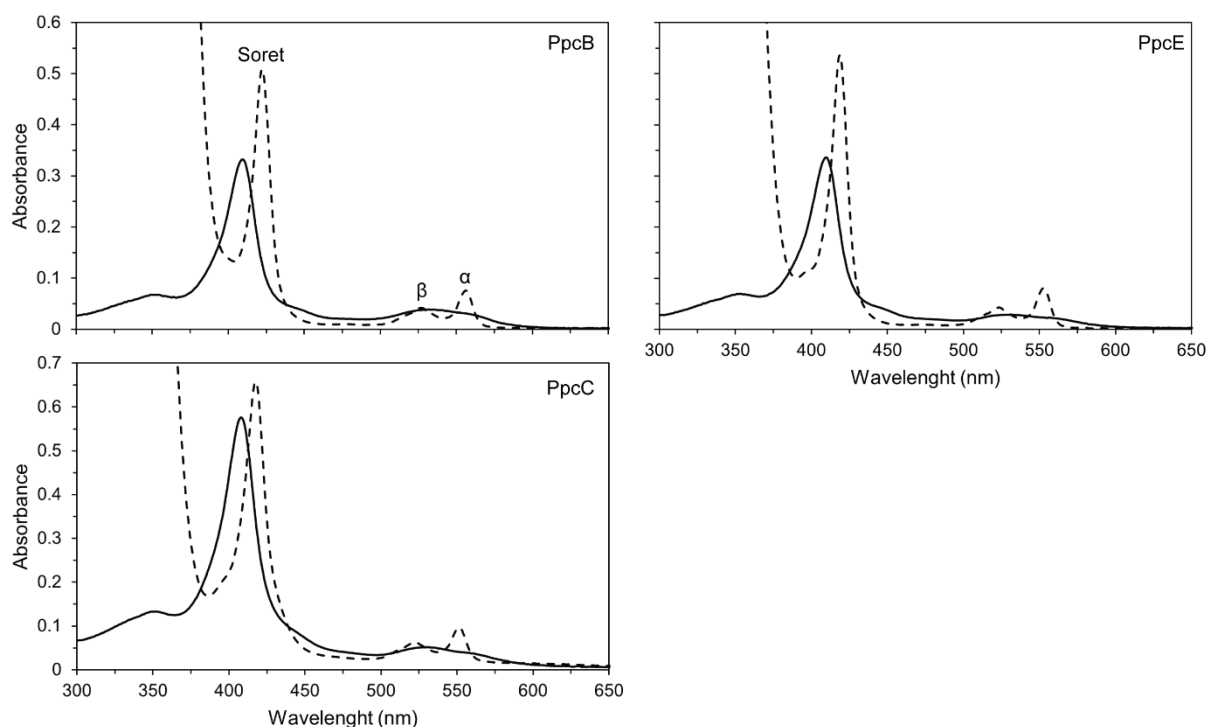


Figure 3.10 - UV-visible spectra of the fully oxidized (solid line) and reduced (dashed line) forms of cytochromes PpcB, PpcC and PpcE from *G. metallireducens*.

The spectral properties of the cytochromes in the UV-visible range indicate the presence of a low-spin hexacoordinated heme [25], but they do not offer conclusive evidence about the nature of the distal heme axial ligand. In some cytochromes His-Met axial coordination of the heme, originates a distinct band at 695 nm in the oxidized spectrum [26]. However, although the presence of this band is a strong indicator of *c*-type hemes axially coordinated by a methionine residue, its absence does not permit to conclude about the nature of the heme's distal axial ligand. In fact, the UV-visible spectral fingerprint for *c*-type hemes with His-Met axial coordination may not always be observable in practice [27-29]. Hence, NMR spectroscopy was used to ensure the correct folding of the proteins and to further confirm the heme axial ligands and heme spin state of the cytochromes. 1D ^1H NMR spectra of cytochromes in the reduced and oxidized state are indicated in Figure 3.11.

Table 3.5 – UV-visible absorptions bands of PpcB-F cytochromes from *G. metallireducens* in the reduced and oxidized states. The data of cytochrome PpcF is also included for comparison.

	Absorption band (nm)					
	Oxidized state			Reduced state		
PpcB	354	409	532	418	523	552
PpcC	351	408	531	417	522	551
PpcE	353	410	531	419	523	553
PpcF	352	410	531	419	523	553

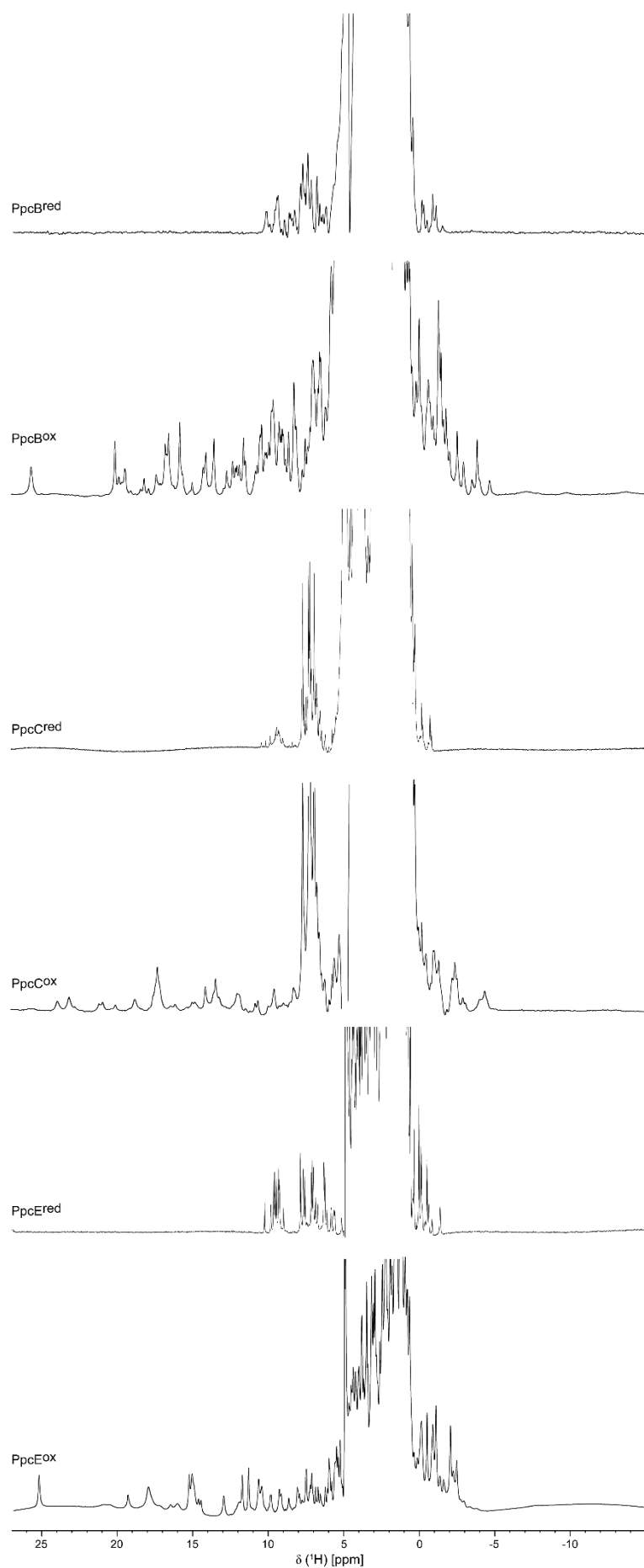


Figure 3.11 - 1D ¹H NMR spectra of cytochromes PpcB, PpcC and PpcE from *G. metallireducens* in the reduced and oxidized forms at 15 °C, 280 mM ionic strength and pH 8.

High-spin cytochromes in the oxidized state show 1D ^1H NMR spectra with extremely broad signals above 40 ppm, which typically correspond to the heme methyls substituents. On the other hand, in low-spin cytochromes, the methyl signals are mostly found in the 8-35 ppm region. In the reduced form, 1D ^1H NMR spectra are also distinct in both spin states, since cytochromes in high-spin show wider spectral regions (commonly from -15 up to 30 ppm) in comparison with the low-spin ones (frequently from -5 to 10 ppm). Hence, 1D ^1H spectra of PpcB, PpcC and PpcE (Figure 3.11) corroborate the fact that these cytochromes are low-spin in both reduced and oxidized states.

Furthermore, in the reduced state is not observable the typical pattern of a distal coordinated methionine that is characterized by three-proton intensity peak at approximately -3 ppm and up to four resolved one proton intensity peaks in the low-frequency region of the spectrum due to the heme ring-current effects [25]. This is in line with what is expected from sequence alignment with the homologous cytochromes (Figure 1.5) that predicts a His-His heme coordination.

3.3.3 Preliminary structure characterization

To initiate the structural characterization of these cytochromes we proceeded to assign the heme substituents proton signals of PpcC and PpcE in the reduced (Table 3.6) state with resource to 2D ^1H -NOESY and 2D ^1H -TOCSY NMR experiments and following the strategy described in section 4.1.8.2.

Table 3.6 - Heme resonance assignment of cytochromes PpcC and PpcE from *G. metallireducens* in the reduced state (pH 8, 15 °C and 250 mM final ionic strength).

	PpcC			PpcE		
Heme substituent	Chemical shift (ppm)					
	Heme I	Heme III	Heme IV	Heme I	Heme III	Heme IV
2 ¹ CH ₃	3.56	4.44	3.61	4.54	4.40	3.84
7 ¹ CH ₃	3.61	4.12	3.13	3.46	4.40	3.46
12 ¹ CH ₃	2.96	3.53	3.40	3.03	3.99/4.29?	3.44
18 ¹ CH ₃	3.35	3.90	3.49	4.12	4.02	2.88
5H	9.47	10.53	9.12	9.25	9.32	9.25
10H	9.30	9.97	9.39	8.98	9.82	9.65
20H	9.54	10.23	9.48	9.57	10.24	9.28
3 ² CH ₃	2.06	1.64	2.12	2.70	1.44	2.37
3 ¹ H	5.74	6.72	5.96	5.58	6.32	5.64
8 ² CH ₃	1.64	2.99	1.61	2.13	2.52	2.90
8 ¹ H	6.36	6.64	6.37	5.85	6.72	6.30

Regarding cytochrome PpcE, while in the reduced state the spectra showed enough quality to complete the assignment of the heme substituents, those obtained in the oxidized state don't. In fact, comparing with the 2D ^1H , ^{13}C -HMQC spectrum of PpcF (Figure 3.12) most of the signals, including those corresponding to the heme groups, are not visible probably because of excessive line broadening. In fact, when the cytochrome is re-reduced all the heme resonances are visible again in the NMR spectra.

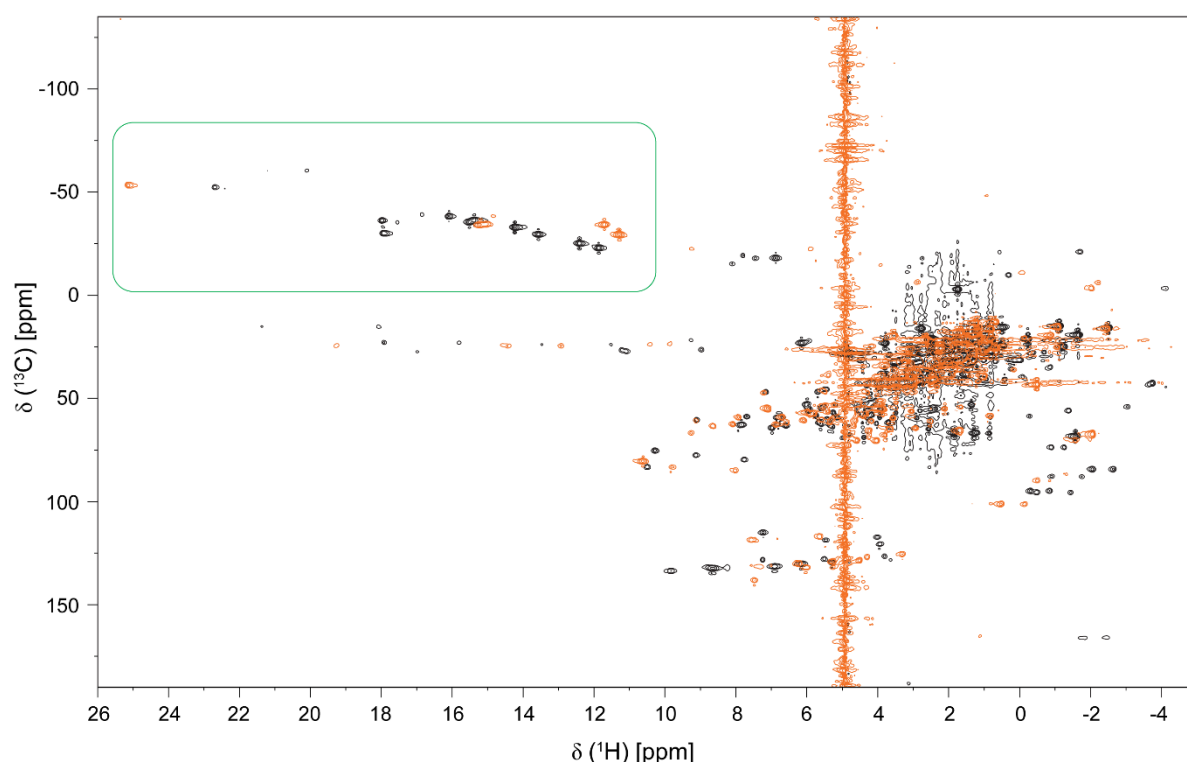


Figure 3.12 – Overlay of 2D ^1H , ^{13}C -HMQC spectra of cytochromes PpcF (black) and PpcE (orange). The green rectangle indicates the heme methyls typical region.

However, the analysis of the NOE connectivities observed in the 2D ^1H -NOESY spectra acquired for PpcE and PpcC indicate that the architecture of the heme core of the PpcA-family is also conserved in these cytochromes (Figure 3.13).

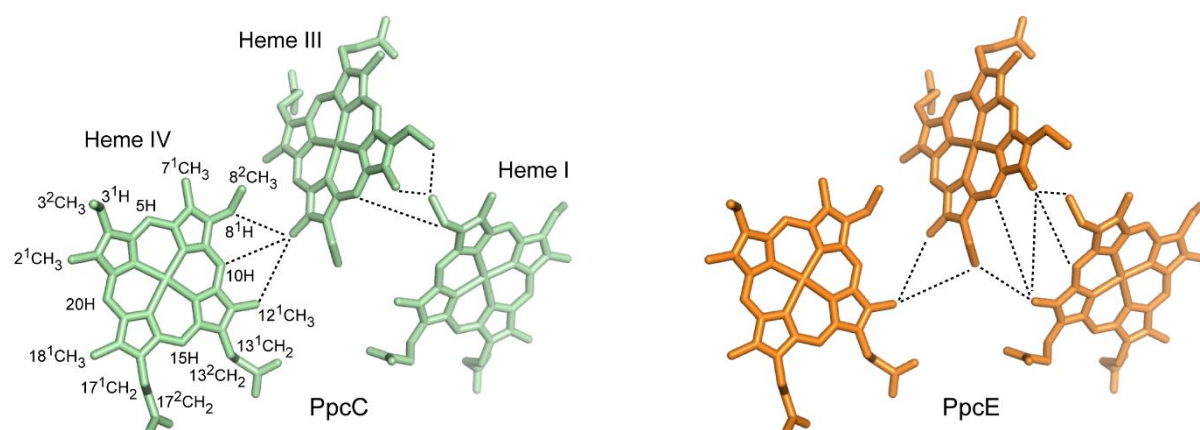


Figure 3.13 - Interheme NOE connectivities observed in the 2D ^1H -NOESY spectra of PpcC and PpcE from *G. metallireducens*. The heme core of PpcA from *G. sulfurreducens* is used as model and the heme substituents are labeled according to the IUPAC-IUB nomenclature for tetrapyrroles [30].

To further investigate the structure of cytochromes PpcB, PpcC and PpcE of *G. metallireducens*, a prediction for each cytochrome was calculated using AlphaFold tool [19]. The models obtained are indicated in Figure 3.14 and superimposed with its homolog in *G. sulfurreducens*.

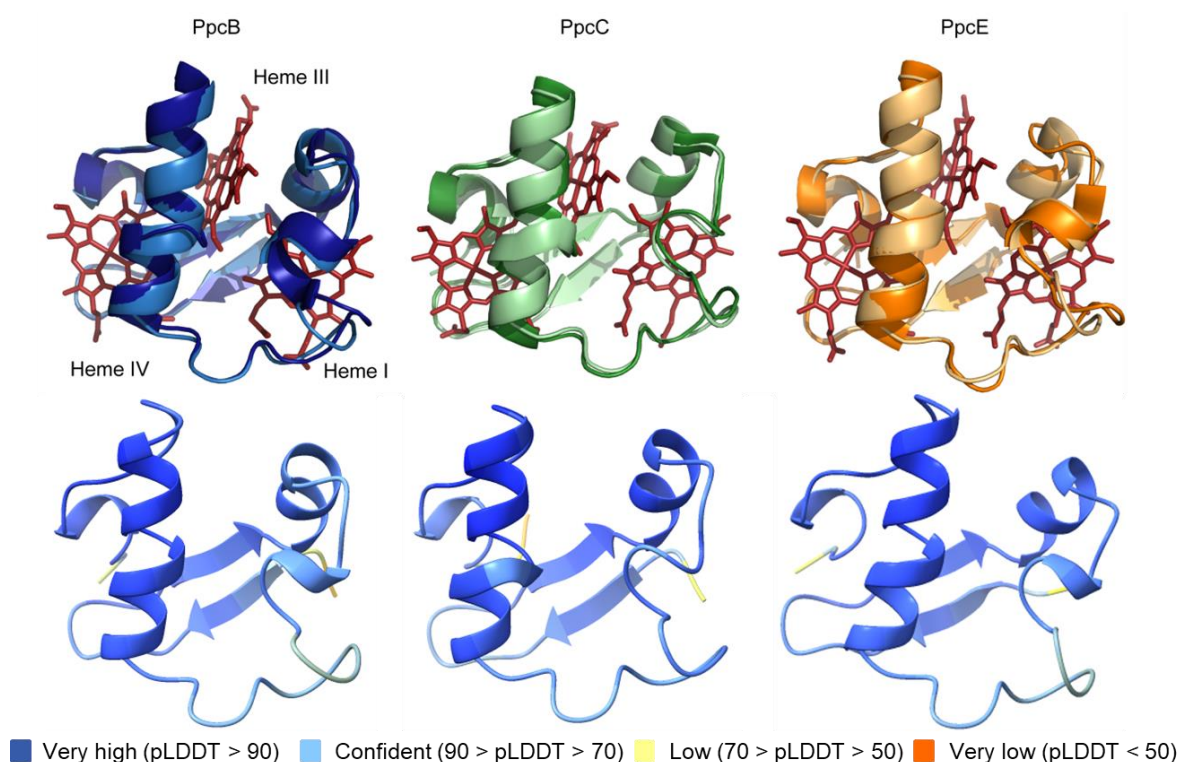


Figure 3.14 – Overlay of cytochromes PpcB, PpcC and PpcE from *G. metallireducens* AlphaFold models with structures determined for its homologs in *G. sulfurreducens* and AlphaFold pLDDT for each model. The structures of PpcB (PDB code 3BXU [1]), PpcC (PDB code 3H33 [3]) and PpcE (PDB code 3H34 [3]) of *G. sulfurreducens* are represented in dark colors while the *G. metallireducens* homologs are represent by the corresponding light color. The molecules are all in the same orientation. Sequence identity percentage between the *G. metallireducens* and *G. sulfurreducens* cytochromes PpcB, PpcC and PpE is 72, 79 and 69%, respectively. Figure produced with PyMOL [22].

The AlphaFold models calculated for each cytochrome also suggest that *G. metallireducens* cytochromes are structurally similar to their homologs from *G. sulfurreducens*. Furthermore, we can observe that, although AlphaFold does not calculate the structure with the heme groups, the heme binding sites and the position of the axial ligands are nearly identical. Hence, the obtained models support the hypothesis that the heme core is conserved among the homologous cytochromes and furthermore that their structure is conserved.

3.3.4 Preliminary thermodynamic characterization

As mentioned previously, the redox potential values determined for cytochromes PpcA and PpcF from *G. metallireducens* have less negative potential values compared to those of the PpcA-family members in *G. sulfurreducens*.

In this Thesis the redox potential value of PpcE from *G. metallireducens* at pH 7 and 8 was also determined using UV-visible potentiometric redox titrations (Figure 3.15).

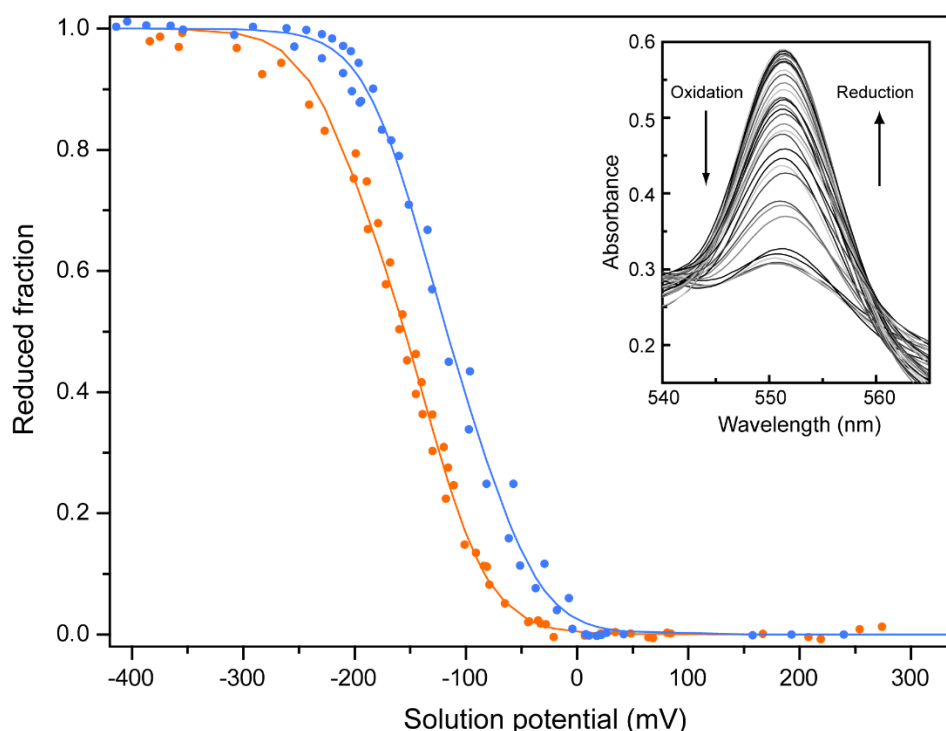


Figure 3.15 - Redox titration curves for cytochrome PpcE from *G. metallireducens* at pH 7.0 and 8.0 (15 °C). The blue and orange symbols refer to pH 7.0 and 8.0, respectively. The lines indicate the results of the fits for the three macroscopic reduction potentials (E_1 , E_2 , and E_3), which are indicated in Table 3.7. The inset represents an expansion of the α -band region at pH 7.0.

The data obtained in Table 3.7 indicates that the working functional redox window of cytochrome PpcE from *G. metallireducens* is more comparable to the cytochromes of the PpcA-family in *G. sulfurreducens*, although more negative at pH 8.0 and less negative at pH 7.0. Compared to PpcA and PpcF from *G. metallireducens* the values are clearly more negative at both pH values.

Table 3.7 - Macroscopic (E_{1-3}) and apparent midpoint (E_{app}) reduction potentials of cytochromes PpcE, PpcA and PpcF from *G. metallireducens* (*Gm*) and PpcA-family cytochromes from *G. sulfurreducens* (*Gs*). The E_{app} values correspond to the point at which the oxidized and reduced fractions are equal. E_1 , E_2 , and E_3 are the macroscopic reduction potentials for the first, second, and third oxidation steps.

Cytochrome	pH 7				pH 8			
	E_{app}	E_1	E_2	E_3	E_{app}	E_1	E_2	E_3
PpcE <i>Gm</i>	-117	-213	-152	-108	-156	-165	-119	-63
PpcA <i>Gm</i> [5]	-78	-132	-84	-8	-93	-150	-95	20
PpcF <i>Gm</i> [6]	-56	-108	-59	10	-64	-125	-66	6
PpcA <i>Gs</i> [1]	-117	-171	-119	-60	-138	-182	-139	-93
PpcB <i>Gs</i> [1]	-137	-185	-140	-84	-143	-192	-145	-103
PpcD <i>Gs</i> [2]	-132	-181	-133	-78	-148	-191	-152	-94
PpcE <i>Gs</i> [2]	-134	-191	-133	-82	-139	-194	-138	-85

The difference in the apparent midpoint reduction potential values of PpcE from *G. metallireducens* at pH 7 and 8 is the highest (39 mV) ever reported for any member of the PpcA-family cytochromes. This indicates that the properties of the individual redox center are strongly modulated by the pH (redox-Bohr effect). The magnitude of the pH dependence of cytochrome PpcE from *G. metallireducens* may indicate that the network of redox-Bohr interactions is clearly different in this cytochrome compared to its homologs and this difference might have some physiological relevance.

2D-EXSY NMR spectra of PpcE in intermediate stages of oxidation collected at different pH values are necessary to determine the oxidation profiles of each heme group and to assist the determination of the individual redox potential values, as well as the properties of the redox-Bohr center. However, as was shown for the 2D ^1H , ^{13}C -HMQC NMR spectrum in the oxidized state (Figure 3.12), also at intermediate levels of oxidation, it was not possible to observe all the necessary signals in the 2D ^1H -EXSY NMR spectra to assist the determination of the individual heme oxidation profiles.

3.4 Conclusions

The expression and purification protocols of cytochromes PpcE and PpcC from *G. metallireducens* were improved. In addition, cytochrome PpcB was produced for the first time.

Cytochrome PpcE demonstrated better results for expression in *E. coli* JCB7123 strain with vector pCK32, induction at OD_{600nm}~1.5 and purification with cation exchange chromatography in 2x5 mL BioScale MiniUnosphere™ S cartridge and an elution gradient of 0-1 M NaCl followed by exclusion chromatography with XK 16/70 Superdex 75 column.

Cytochrome PpcC revealed the best results when its expression was performed with *E. coli* JM109 strain with vector pVA203, induction at OD_{600nm}~1.5 and purification with cation exchange chromatography 2x5 mL HiTrap SP HP cartridges and an elution gradient of 0-300 mM NaCl followed by exclusion chromatography with XK 16/70 Superdex 75 column.

The referred expression and purification optimizations culminated in an increase of the expression yield from 0.03 to 0.63 mg/L in PpcE and from 0.05 to 0.54 mg/L of cell culture in PpcC.

Cytochrome PpcB expression and purification conditions showed better results for expression in *E. coli* Tuner strain with pVA203 vector with induction at OD_{600nm}~1.5 and a first chromatographic step with cation exchange chromatography in 2x5 mL BioScale MiniUnosphere™ S cartridges followed by molecular exclusion chromatography in XK 16/70 Superdex 75 column. PpcB was obtained with an expression yield of 3 mg/L of cell culture.

1D ¹H NMR spectra acquired for cytochromes PpcB, PpcC and PpcE from *G. metallireducens* demonstrated that all the heme groups of all proteins are axially coordinated by two histidine residues (His-His) and are low spin in both reduced and oxidized states. Assignment of the heme substituents signals in 2D ¹H-NOESY and 2D ¹H-TOCSY NMR spectra acquired for PpcE and PpcC in the reduced state indicated that the heme core architecture of the *G. sulfurreducens* PpcA-family remains conserved in these cytochromes. Alphafold tool was used to predict the structure of PpcB, PpcC and PpcE from *G. metallireducens* and the proposed models were compared with the determined structures of its homologs in *G. sulfurreducens* which further confirmed that the structures remain conserved.

The preliminary thermodynamic characterization of cytochrome PpcE shows unique properties compared to those obtained for homologous cytochromes either in *G. metallireducens* or *G. sulfurreducens*. In the first case, the reduction potential values are clearly more negative. In the second case they are much more negative at pH 8.0 but less negative at pH 7.0. In both cases the difference in the apparent midpoint reduction potential at pH 7 and 8 is the highest ever reported, indicating that the redox-Bohr center is strongly affected by the pH.

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3. Optimization of the production of cytochromes P₄₅₀B-E from *Gm* and preliminary characterization

**BIOCHEMICAL AND BIOPHYSICAL
CHARACTERIZATION OF CYTOCHROME PpcF
FROM *G. metallireducens*¹**

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4. Biochemical and biophysical characterization of cytochrome PpcF from *G. metallireducens*

4.1 Introduction

G. metallireducens genome encodes for 65 multiheme cytochromes, which are numerically comparable to the 73 multiheme cytochromes encoded by the genome of *G. sulfurreducens* [1]. However, genetic and proteomic studies indicate that the essential *c*-type cytochromes for cellular growth in the presence of insoluble and soluble electron acceptors are distinct in the two bacteria [2]. This also extends to the well-conserved PpcA-family of *c*-type triheme periplasmic cytochromes that are amongst the most abundant in *Geobacter* species [3, 4].

PpcA-family is conserved in both bacteria. The members of these families have a crucial role in the electron flow towards extracellular electron acceptors, but are unlikely to be simultaneously involved in the same respiratory pathway, as suggested by proteomic and genetic studies [5-7]. The members of the PpcA-families are named PpcA-E in *G. sulfurreducens* and PpcA-C, PpcE and PpcF in *G. metallireducens*. The PpcF designation derives from its low amino acid sequence identity with cytochrome PpcD from *G. sulfurreducens* [8]. Hence, PpcF stands out in the PpcA-family of *G. metallireducens*, which together with the evidences that the cytochrome is crucial for nitrate respiration by exchanging electrons with the nitrate reductase enzyme [5], makes it a very interesting target for functional studies. A preliminary characterization of PpcF from *G. metallireducens* was performed prior to this Thesis. Firstly, the molecular mass of the expressed protein was confirmed by mass spectroscopy (9737.13 Da) and the molar extinction coefficient was determined ($87.4 \text{ mM}^{-1}\text{cm}^{-1}$). UV-visible spectroscopy showed that the heme groups of PpcF are low spin both in the reduce and oxidized states, a feature that was further confirmed by NMR spectroscopy. Furthermore, the analysis of the hemes NOE connectivities showed that the heme core structure is similar to those of the PpcA family cytochromes in *G. sulfurreducens*. The reduction potential values of PpcF were determined at pH 7 and 8 (-56 mV and -64 mV *versus* the SHE, respectively). Lastly 2D- ^1H NMR exchange spectroscopy was used to determine the order of oxidation of the heme groups in PpcF: IV-I-III.

4.2 Materials and Methods

4.2.1 Amino acid sequence analysis and cloning of cytochrome *ppcF* gene

Genomic DNA from *G. metallireducens* GS-15 was kindly provided by Prof. D. R. Lovley (University of Massachusetts, Amherst). The *ppcF* gene sequence (GenBank accession number ABB30578) was identified by searching the *G. metallireducens* genome database on Kyoto Encyclopedia of Genes and Genomes Web site (www.genome.jp/kegg/genome.html), under the accession number T00295. The gene sequence was analyzed using the SignalP server [9] to predict the signal peptide cleavage, which is located after the residue Ala²².

DNA fragment coding for the mature sequence of cytochrome PpcF was amplified from the *G. metallireducens* GS-15 genomic DNA with primers containing restriction sites for the enzymes NotI (5'-CACATGCGGCCGCGCCGACGTATTTGAATTC-3') and HindIII (5'-GCCCCTACCGCAGGTTCAAGCTTTTACTTCTTGTGGCAG-3'). Fragment size (305bp) was confirmed by 0.8% agarose gel electrophoresis. The DNA fragment was digested with the restriction enzymes NotI and HindIII and cloned into vector pVA203 [15, 20]. The resulting plasmid was designated pCS0335. Colonies were screened by colony PCR with primers (5'-GGCTCGTATGTTGTGTGGAA-3' and 5'-AAGGGAAGAAAGCGAAAGGA-3') and then grown in liquid LB medium supplemented with AMP (100 µg/mL) for plasmid extraction and sequencing (STABvida). *Escherichia coli* strain DH5α was used for cloning and *E. coli* strain BL21(DE3), co-transformed with plasmid pEC86, was used for protein production. Primers were purchased from Invitrogen, restriction enzymes and T4 DNA ligase from Fermentas. All PCR products were purified using Wizard PCR Preps DNA Purification System (Promega). Digested vector and plasmids were purified using E-gel Electrophoresis System (Invitrogen) and NZYMiniprep kit (NZYTech), respectively. Phusion High-Fidelity DNA polymerase (Finnzymes) was used for amplification from genomic DNA and Taq DNA polymerase (VWR) for colony PCR.

4.2.2 Heterologous expression of cytochrome PpcF

The heterologous expression of PpcF was performed using *E. coli* strain BL21(DE3), harboring the plasmid pEC86 [10], which is a derivative from the pACYC184 containing the gene cluster needed to the cytochrome *c*, *ccmABCDEFGHIH*. These competent cells are then co-transformed with the expression plasmid, described in the previous section, containing the gene sequence.

Transformed *E. coli* cells were grown aerobically in 2x Yeast extract – Tryptone (2xYT) liquid medium supplemented with 34 µg/mL CLO and 100 µg/mL AMP, both from NZYTech. Colonies were selected and grown in 50mL 2xYT media (also supplemented with 34 µg/mL CLO and 100 µg/mL AMP) to a OD_{600nm}~1.8-2, at 30 °C, 200 rpm, overnight. 20% of this culture

was transferred to 1L of 2xYT media (34 $\mu\text{g/mL}$ CLO and 100 $\mu\text{g/mL}$ AMP) and grown till they reach an $\text{OD}_{600\text{nm}} \sim 1.5$, at 30 °C and 200 rpm.

All the steps described above are analogous to produce labeled or unlabeled proteins. From this step forward, production of unlabeled and labeled proteins was carried out through two different processes (Figure 4.1) and are described in the following sections.

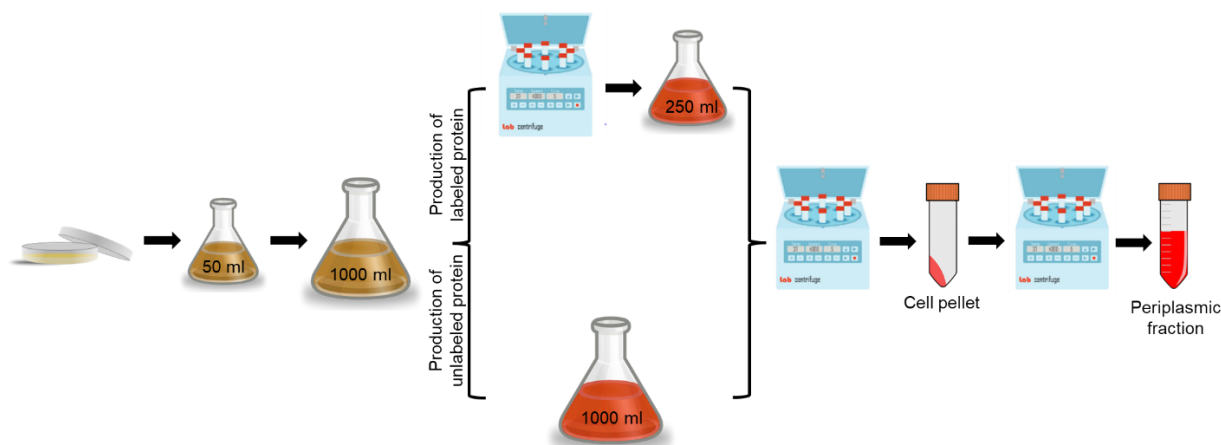


Figure 4.1 - Production and isolation scheme for unlabeled and ^{13}C and/or ^{15}N labeled proteins.

4.2.3 Natural abundance production of cytochrome PpcF

At this stage, protein expression was induced with 10 μM IPTG from NZYTech and incubated overnight at 30 °C and 160 rpm. Then, cells were harvested and centrifuged at 6400 $\times g$ for 20 min at 4 °C, to isolate the periplasmic fraction.

4.2.4 Isotopically labeled (^{15}N and/or ^{13}C) production of cytochrome PpcF

After the cells reach the mid-exponential phase ($\text{OD}_{600\text{nm}} > 1.5$), cultures were harvested by centrifugation at 6400 $\times g$, 30 min. The resultant cell pellet was washed twice with 250 mL of a salt solution containing 43 mM NaCl (Panreac), 240 mM Na_2HPO_4 (VWR Chemicals) and 110 mM KH_2PO_4 (Riedel-de-Haën). Afterwards, cells were resuspended in 250 mL minimal media composed by 34 $\mu\text{g/mL}$ CLO, 100 $\mu\text{g/mL}$ AMP, 8.6 mM NaCl, 48 mM Na_2HPO_4 (VWR), 22 mM KH_2PO_4 (Panreac), 20 mg/L biotin (Sigma), 20 mg/L thiamine (Merck), 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Panreac), 0.1 mM CaCl_2 (Sigma-Aldrich), 5 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (VWR), 10 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck), 1 mM of the heme precursor δ -aminolevulinic acid (Merk), 0.2 g/L $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories, Inc.) as nitrogen source and 0.4 g/L D-glucose ^{12}C (VWR) or 2 g/L ^{13}C -Glucose (Sigma) as carbon source. Then, the cultures were incubated at 30 °C and 180 rpm, during 1h. Protein expression was induced with 100 μM IPTG. After induction, cells were left overnight, at 30 °C and 160 rpm.

4.2.5 Isolation of the periplasmic fraction

To isolate the periplasmic fraction, cells were centrifuged at 6400 $\times g$ for 20 min at 4 °C. The cell pellet was gently resuspended in 30 mL of lysis buffer, per liter of initial cell culture, constituted by: 20 % sucrose (VWR), 200 mM Tris-HCl (NZYTech) pH 8, 0.5 mM EDTA (Amresco) and 0.5 mg/mL lysozyme (Fluka). The suspension was incubated at room temperature during 15 min, then 15 min on ice with gently shaking and finally centrifuged at 14700 $\times g$, 20 min, at 4 °C. The resulting supernatant was ultracentrifuged at 44000 $\times g$, 1 h, at 30 °C. The resulting supernatant was dialyzed (MWCO 3500) against 10 mM Tris-HCl pH 8.

4.2.6 Protein purification

PpcF was purified using first a cation exchange chromatography and then a molecular exclusion chromatography. Dialyzed protein was loaded in a cation exchange 2x5 mL Bio-Scale Mini UNOsphere S cartridges (BioRad), equilibrated with 10 mM Tris-HCl pH 8 and the cytochrome was eluted with a 0-300 mM NaCl, 150 mL length gradient in 10 mM Tris-HCl, at a flow rate of 1 mL/min. The collected red fractions were concentrated to 1 mL and injected in a XK 16/70 column (GE Healthcare) packed with Superdex 75 (GE Healthcare), with a molecular weight range between 3 and 70 kDa, equilibrated with 100 mM sodium phosphate buffer pH 8, and eluted at a flow rate of 0.5 mL/min. Both chromatography steps were performed on an ÄKTA Pure System (GE Healthcare).

4.2.7 Protein purity evaluation and quantification

The protein purity was evaluated by SDS-PAGE gel (5% acrylamide stacking gel and 15% acrylamide running gel) using a Mini-PROTEAN® Electrophoresis System (Bio-Rad). Samples were prepared containing SDS-PAGE Sample Loading Buffer (NZYTech) in equal volume and were run at 120 V during 90 min. Precision Plus Protein™ Dual Xtra Prestained Protein Standards (Bio-Rad) marker with MW range 2 to 250 kDa was used. Protein bands were revealed with TMBZ (Acros Organics) and/or BlueSafe (NZYTech).

UV-visible absorption spectra in the oxidized and reduced states acquired at room temperature with quartz cuvettes with 1 cm path length on a Ultraspec 2100pro spectrophotometer (Amersham Biosciences, Switzerland) were used for protein quantification. PpcF was fully reduced by adding crystals of sodium dithionite (Thermo Fisher Scientific) to the sample. Protein quantification was determined using the molar extinction coefficient for reduced PpcF at 553 nm ($87 \text{ mM}^{-1} \text{ cm}^{-1}$) [8].

4.2.8 NMR studies

4.2.8.1 Sample preparation

All NMR spectra were acquired in a Bruker Avance III 600 spectrometer equipped with a triple-resonance cryoprobe (TCI) at 15 or 25 °C and processed using TOPSPIN (BrukerBiospin, Karlsruhe, Germany). ^{13}C and ^{15}N chemical shifts were calibrated through indirect referencing [11]. ^1H chemical shifts are referenced to DSS at 0 ppm by using the residual water signal as an internal secondary reference, as previously described [12].

To assist backbone, side chain and heme assignments PpcF samples were prepared in the concentration range 0.5 and 1.7 mM in sodium phosphate buffer 45 mM (final ionic strength of 100 mM) pH 7.1 either in 92% H_2O / 8% $^2\text{H}_2\text{O}$ for assignment of backbone and side chains or in $^2\text{H}_2\text{O}$ (99% atom) for heme substituents' assignment in the oxidized state. The pH values of the samples were checked with a glass micro electrode and were not corrected for isotope effects. To assist the heme signal resonance assignments in the oxidized state, 2D ^1H -NOESY (80 ms mixing time) and TOCSY (45 ms mixing time) over a spectral width of 41 kHz and 2D $^1\text{H},^{13}\text{C}$ -HMQC (41 and 250 kHz in ^1H and ^{13}C dimensions, respectively) NMR spectra were acquired collecting 4096 (t_2) $\times$ 512 (t_1) data points and 136 scans per increment for 2D ^1H -NOESY and 2D ^1H -TOCSY and 4096 (t_2) $\times$ 256 (t_1) data points and 420 scans per increment for 2D $^1\text{H},^{13}\text{C}$ -HMQC. For backbone and side chain assignments a set of experiments were acquired for both (1) ^{13}C , ^{15}N -labeled PpcF or (2) natural abundance PpcF: (1) 2D $^1\text{H},^{13}\text{C}$ -HSQC, 3D-HNCACB, 3D-HN(CO)CACB, 3D-HNCA, 3D-HN(CO)CA, 3D-HCC(H)-TOCSY, 3D-HC(C)H-TOCSY and 3D-NOESY-HSQC and (2) 2D ^1H -TOCSY (20 and 60 ms) and 2D ^1H -NOESY (80 ms).

To monitor the individual heme oxidation patterns, samples (80 μM) were prepared in 80 mM sodium phosphate with NaCl (250 mM final ionic strength) in $^2\text{H}_2\text{O}$, at different pH values and covering the pH range 6.0 – 8.5. The fully reduced samples were obtained by adding catalytic amounts of the enzyme hydrogenase from *Desulfovibrio vulgaris* (Hildenborough), as previously described [13]. Partially oxidized samples were obtained by first removing the hydrogen from the fully reduced sample with argon and then adding controlled amounts of air into the NMR tube with a Hamilton syringe. Each 2D ^1H -EXSY NMR spectrum was acquired with a mixing time of 25 ms, collecting 2048 (t_2) $\times$ 256 (t_1) data points and 256 scans per increment with the sample poised at different degrees of oxidation. The desired partial oxidation level of the samples was selected by the inspection of 1D ^1H NMR spectra. 1D ^1H NMR spectra with water pre-saturation, collecting 32k data points and covering a spectral width of 24.6 kHz, were acquired before and after each 2D ^1H -EXSY NMR spectrum, to check for any changes in the sample oxidation level.

To assign the heme α -substituents of PpcF in the fully oxidized state, a set of 2D NMR experiments was acquired with either natural abundance or ^{13}C labeled samples. The 2D $^1\text{H},^{13}\text{C}$ -HSQC acquired for the natural abundance sample collected 4096 (t_2) $\times$ 256 (t_1) data

points, with 520 scans per increment, covering a sweep width of 24.6 and 52.8 kHz in the ^1H and ^{13}C dimension, respectively. To distinguish the signals from the polypeptide chain from the heme signals, 2D ^1H , ^{13}C -HSQC spectrum was acquired with ^{13}C labeled sample, collecting $2048 (t_2) \times 256 (t_1)$ data points, covering the same sweep widths of the spectrum acquired for the natural abundance sample, but only with 16 scans per increment. 2D ^1H -NOESY spectrum was acquired with a mixing-time of 80 ms, collecting $4096 (t_2) \times 512 (t_1)$ data points, with 136 scans per increment, covering a sweep width of 24.6 kHz. 2D ^1H -TOCSY spectrum was acquired with a mixing-time of 45 ms while maintaining the above-mentioned conditions for the 2D ^1H -NOESY spectrum. Finally, to evaluate the temperature dependence of the heme methyl signals, additional 1D ^1H NMR spectra were measured between 15 and 35 °C.

4.2.8.2 Assignment of the heme substituents in the reduced and oxidized states

In the reduced state, the first step to assign the heme substituents signals is the analysis of the connectivities between a thioether methine (3^1H or 8^1H) and a thioether methyl group (3^2CH_3 or 8^2CH_3) in the 2D ^1H -TOCSY NMR spectrum, as depicted in Figure 4.2. Indeed, these are the only protons that are part of the same spin system and, therefore can be easily detected in such NMR experiments.

On the other hand, 2D ^1H -NOESY experiments allow detecting spatial correlation between nuclei that are typically closer than 5 Å. Thus, as also depicted in Figure 4.2, meso protons present a characteristic pattern of short-range intraheme connectivities: protons H15 are not connected to either methyl groups or thioether substituents; protons H20 are connected to two heme methyls (2^1CH_3 and 18^1CH_3); and the only ambiguity arises from H5 and H10 protons, which both present connectivities with a thioether methine, a thioether methyl and one heme methyl group. This ambiguity was solved by observing the connectivities between one of the heme methyls near the H20 protons (2^1CH_3 and 18^1CH_3) with the closest thioether methyl (3^2CH_3), which were unequivocally assign in the 2D ^1H -TOCSY. This allowed connecting H20 and H5 sides of each heme. The heme methyls 7^1CH_3 are part of H5 side and also show connectivities with thioether groups (8^1H and 8^2CH_3), which are in H10 side. After the identification of these three heme sides, H15 protons were identified by observing the connectivities between cross-peaks that connect H15 and 12^1CH_3 or 18^1CH_3 protons (Figure 4.2).

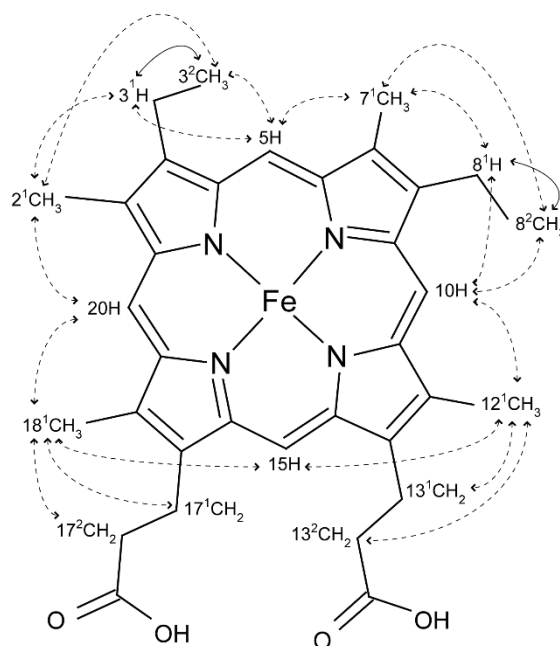


Figure 4.2 - Diagram of heme *c* showing the connectivities used to assign the heme substituent signals. The typical through bond connectivities observed in the 2D ¹H-TOCSY (except for heme propionates) are indicated by solid arrows, whereas the dashed ones indicate the typical NOE connectivities that arise from the 2D ¹H-NOESY spectra used to assign the heme substituents. Heme substituents are numbered according to the IUPAC-IUB nomenclature [14].

In the paramagnetic oxidized state, in addition to the ring-current effects, the intrinsic (from own heme) and the extrinsic (from neighboring hemes) paramagnetic contribution due to the presence of unpaired electrons strongly contributes to the final observed chemical shift of the heme substituents, making their assignment more complex. In fact, in the oxidized form the same type of signals is differently affected by the paramagnetic centers, show different levels of broadness and are spread all over the entire NMR spectral width. Therefore, the chemical shifts of the heme substituents in the oxidized form are completely different in comparison with those observed in the fully reduced proteins. Consequently, a different NMR assignment strategy was used to assign the heme signals in the oxidized form. In this case, 2D ¹H,¹³C-HMQC NMR experiment is very useful to map some of the heme substituent signals because typical ¹H, ¹³C regions can be identified. The propionates αCH₂ protons (17¹CH₂ and 13¹CH₂) are identified in 2D ¹H,¹³C-HMQC NMR spectrum, whereas the intraheme connectivities with the propionates βCH₂ (17²CH₂ and 13²CH₂) are obtained from the analysis of 2D ¹H-TOCSY spectrum and then confirmed in 2D ¹H,¹³C-HMQC NMR spectrum at the typical region of propionates βCH₂. Afterwards, in 2D ¹H-NOESY spectrum we identified the cross peaks of each propionate proton with those of the closest heme methyl (18¹CH₃ and 12¹CH₃).

4.2.8.3 Backbone and side chain resonance assignment methodology

In the protein backbone assignment, at first, a random signal from the 2D ^{15}N , ^{13}C -HSQC spectrum was chosen, and using the ^{15}N and ^{13}C chemical shifts, its path was followed into the 3D spectra. All the 3D spectra recorded were aligned with the HSQC and between themselves, so that all ^{15}N , ^{13}C and ^1H chemical shifts variations could be followed simultaneously. For the assignment of the C_α of each amino acid, the HNCA spectrum was used to find the $\text{C}_{\alpha i}$ and $\text{C}_{\alpha i-1}$ chemical shifts, and to distinguish between them, the HN(CO)CA was used, since only the $\text{C}_{\alpha i-1}$ appears in this spectrum. This assignment can be also confirmed in the HNCACB and HN(CO)CACB. At the same time, for the assignment of the C_β , the HNCACB spectrum was used to find the C_β and $\text{C}_{\beta i-1}$ chemical shifts, and to distinguish between them, the HN(CO)CACB was used, since only the $\text{C}_{\beta i-1}$ signal appears in this spectrum. After getting all the possible information from a single HSQC signal, we then navigate either to its following amino acid or to its previous one. In order to navigate to any following amino acid, we used the HN(CO)CA spectrum and by fixing the $\text{C}_{\alpha i}$, and varying the ^{15}N dimension, it was possible to find correspondent peaks (peaks with similar ^{13}C chemical shift, but different ^{15}N and ^1H chemical shift). These new signals corresponded to an amino acid, placed ahead the one we started from. That means that the signals founded were in fact the C_α from the first amino acid being seen as a previous C_α .

A similar strategy can be followed to navigate to any previous amino acid. For that, we used the HNCA spectrum and fixed the chemical shift of $\text{C}_{\alpha i-1}$. The new signals founded corresponded to the previous amino acid itself, meaning that the C_α previously seen as $\text{C}_{\alpha i-1}$, was now seen as a C_α belonging to the amino acid itself.

By continuously using the method above described (peaking a HSQC peak and navigate to its previous and following amino acids), the assignment of the protein backbone was obtained (Figure 4.3).

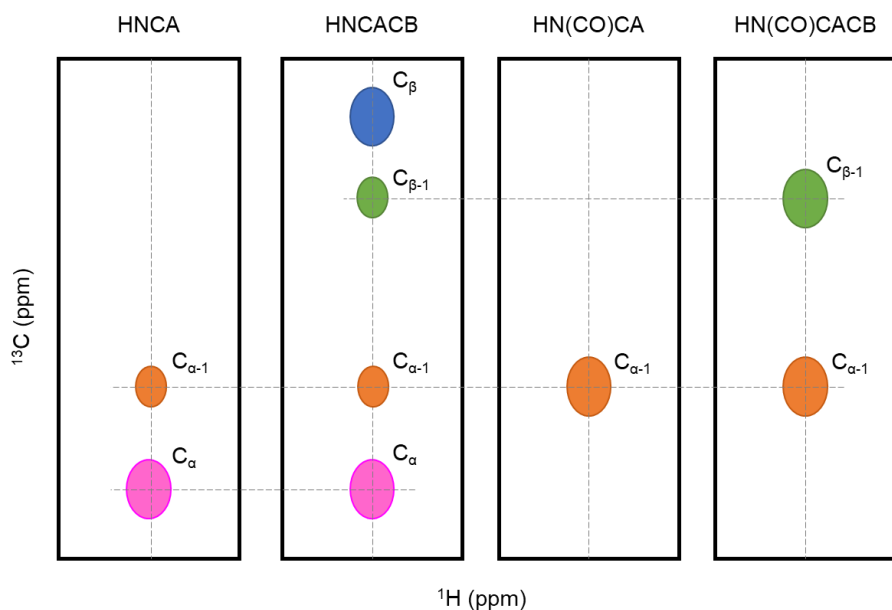


Figure 4.3 - Scheme of the observable signals in 3D-HNCA, 3D-HNCACB, 3D-HN(CO)CA and 3D-HN(CO)CACB NMR spectra in ^1H and ^{13}C coordinates for a given ^{15}N chemical shift.

After identification of the ^1H , ^{15}N , $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ resonances for a given amino acid, the known $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ chemical shifts from the backbone assignment are used as a starting point to identify the remaining aliphatic ^1H and ^{13}C signals in 3D HCC(H)-TOCSY, which are independently confirmed by analysis of the 2D ^1H , ^{13}C -HSQC. In the case of cytochrome PpcF, it was also necessary to acquire 3D-NOESY spectra to complete the assignment.

4.2.8.4 Assignment of the heme α -substituents

As mentioned above, the NMR signatures of the heme methyl substituents are quite distinct in the reduced and oxidized states. Hence, during a redox titration, the chemical shift variation of these signals between the two states can be monitored through 2D ^1H -EXSY NMR experiments. Thus, starting from the fully reduced protein, as the protein oxidation progresses, the heme methyl chemical shifts will be different in each oxidation state until they reach their final position in the fully oxidized sample. Thus, 2D ^1H -EXSY NMR spectra can be used to follow the assignment of those signals through the different oxidation stages.

However, this strategy is often not enough to complete the assignment. Hence, the most straightforward assignment methodology combines the analysis of 2D ^1H , ^{13}C -HSQC NMR spectra recorded for an unlabeled sample and for a ^{13}C labeled sample exclusively in its polypeptide chain (Figure 4.15). Since the heme precursor (δ -aminolevulinic acid) added into the minimal media is not labeled, the heme signals are not observable in spectra acquired with a relatively small number of accumulations for the labeled sample. Thereby, by simple comparison of the two NMR spectra, the heme substituents signals and those of the polypeptide chain can be easily discriminated. Then, the assignments of the heme substituents

were identified by the analysis of the intraheme TOCSY and NOESY connectivities, as previously described [15-17]. Also, the dispersion of the heme α -substituents NMR signals is more arbitrary in the oxidized state than in the reduced state. Therefore, the exclusive use of 2D ^1H -TOCSY and 2D ^1H -NOESY spectra renders the assignment in the oxidized state much more challenging and is often insufficient. In this case, 2D ^1H , ^{13}C -HMQC spectrum is an important complement. Indeed, since the dipolar shifts of the carbon nuclei attached to the pyrrole β -carbons are very small and, in contrast with the protons bonded to them, their Fermi contact shifts are directly proportional to the spin density on the pyrrole β -carbons, the different types of heme α -substituents can be found in specific regions of the 2D ^1H , ^{13}C -HMQC spectrum (Figure 4.4) [18, 19].

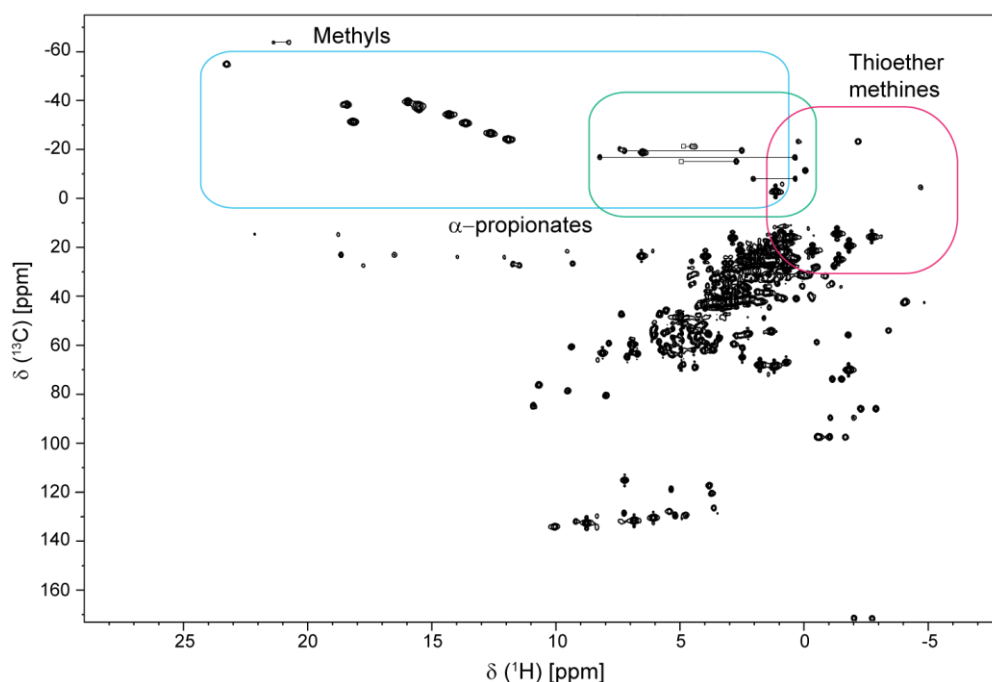


Figure 4.4 - 2D ^1H , ^{13}C -HMQC spectrum of oxidized PpcF (25 °C and pH 7) showcasing the typical regions for the signals of the heme α -substituents.

In particular, the α -propionate groups 13^1CH_2 and 17^1CH_2 are easy to identify since the two protons of these spin systems share the same ^{13}C chemical shift (see straight lines in Figure 4.4). In this section, the assignment of the heme IV α -substituents is illustrated. Following the identification of the α -propionates candidates (13^1CH_2 and 17^1CH_2) in the 2D ^1H , ^{13}C -HMQC spectrum, they can be independently confirmed by the analysis of the connectivities between each pair of protons in the 2D ^1H -TOCSY (Figure 4.5). Then, the 2D ^1H -NOESY spectrum is used to identify the connectivities between each propionate group and the closest heme methyl (12^1CH_3 and 18^1CH_3 – Figure 4.5).

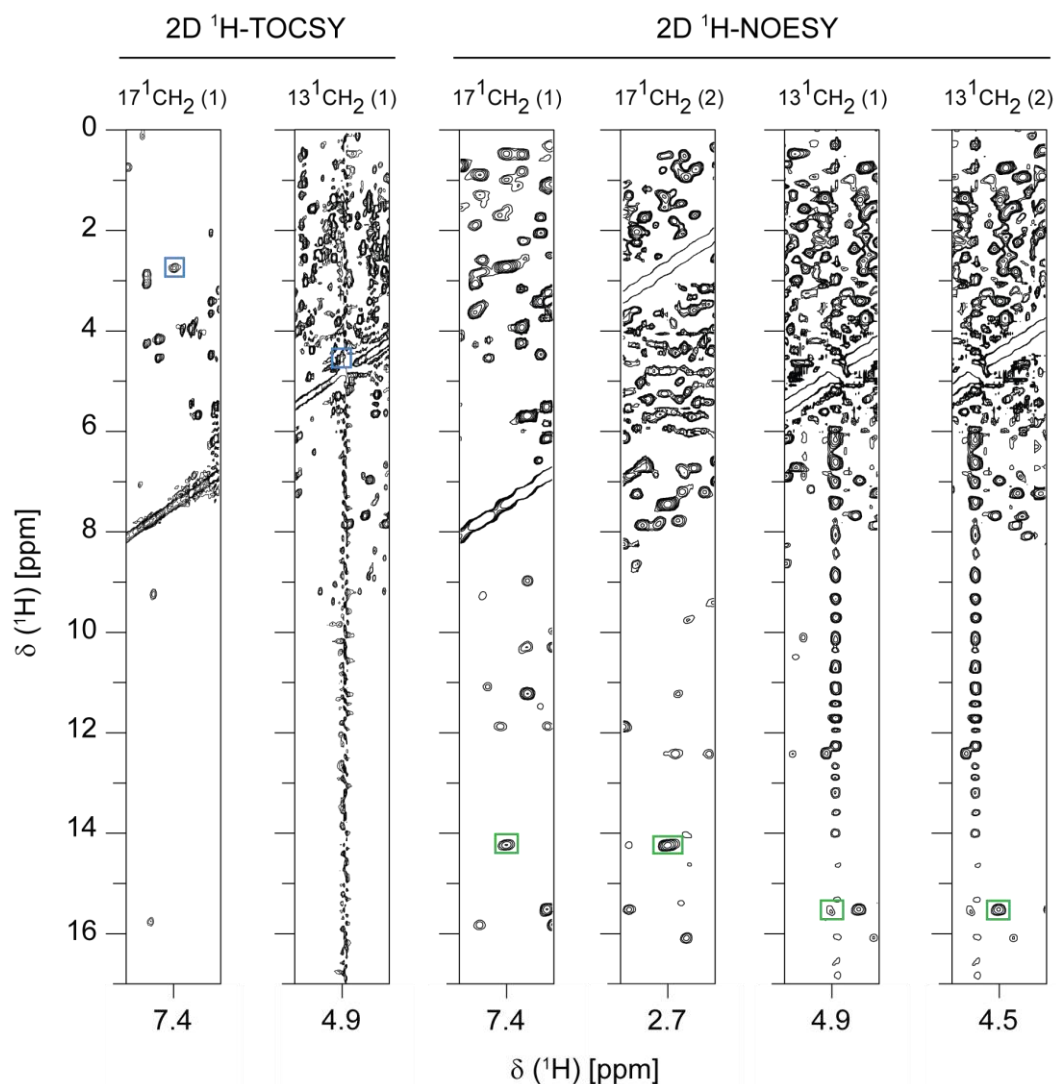


Figure 4.5 - Expansions of the 2D ^1H -TOCSY and 2D ^1H -NOESY spectra of oxidized PpcF (pH 7 and 25 °C). The blue squares highlight the connectivities between the two protons of each α -propionate of heme IV in the 2D ^1H -TOCSY spectrum. The green squares highlight the connectivities between the α -propionate protons and the heme methyls 12^1CH_3 and 18^1CH_3 .

These two heme methyls can be easily distinguished by checking their NOESY connectivities. The 12^1CH_3 heme methyl has no NOESY connectivities with other heme methyls, whereas 18^1CH_3 has a NOESY connectivity with the methyl 2^1CH_3 (Figure 4.6).

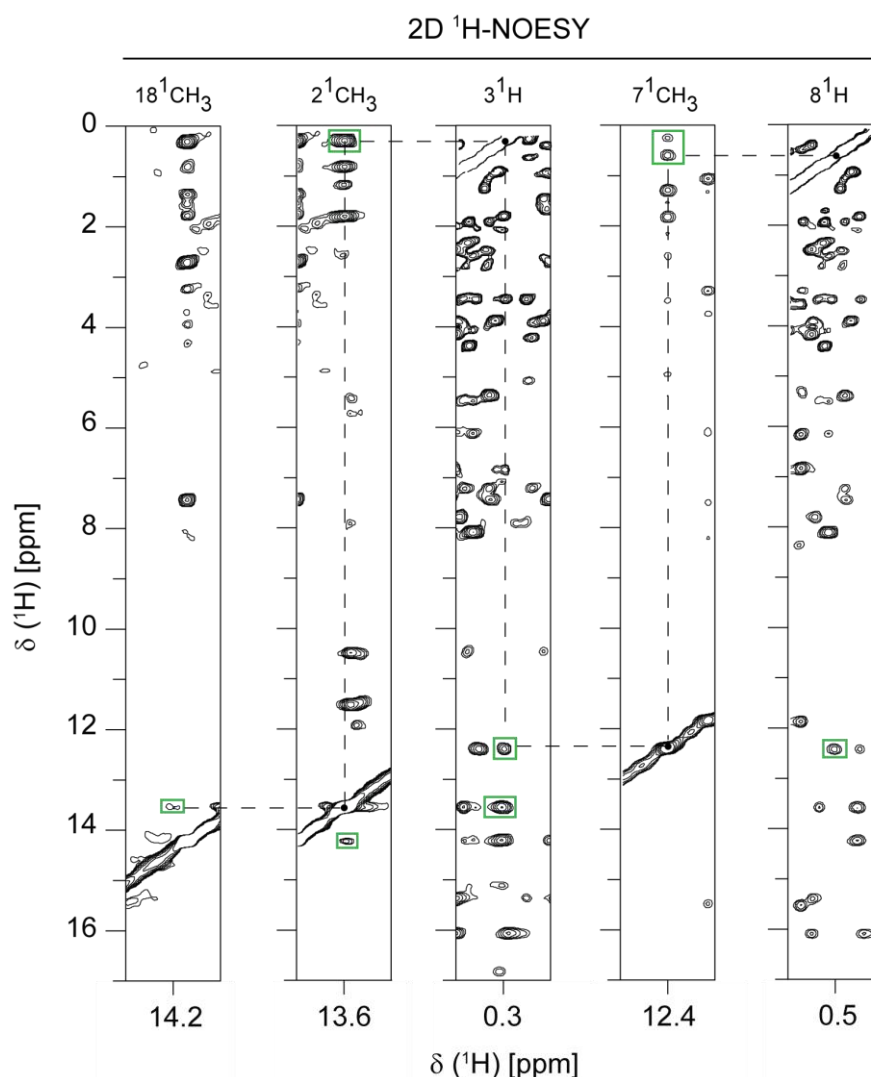


Figure 4.6 - Expansions of the 2D ^1H -NOESY spectrum of oxidized PpcF (pH 7 and 25 °C). The green squares highlight the NOESY connectivities between the different heme α -substituents. The dashed lines represent the heme α -substituents assignment path in a clockwise direction, starting from the heme methyl 18^1CH_3 .

After a proper distinction between 12^1CH_3 and 18^1CH_3 , the 2D ^1H -NOESY spectrum can be used to assign the other heme α -substituents using well established assignment strategies [20, 21] (Figure 4.6).

4.2.9 Circular dichroism spectroscopy

Circular dichroism experiments were performed on a JASCO J-810 spectropolarimeter equipped with a Peltier thermostat cell support. CD spectra were acquired for 70 μM protein samples at pH 8.0. The temperature was controlled to ± 0.1 °C. CD spectra were the average of four scans obtained by collecting data at 0.5 nm intervals and scan rate of 50 nm per minute from 200 to 260 nm. After recording a CD spectrum at 25 °C, the thermal unfolding with a linear temperature increase at 3 °C min^{-1} (from 25 to 95 °C) was followed by recording the

ellipticity variation at 235 nm. The CD spectra were recorded at 95 °C and after cooling back the sample to 25 °C. All spectra and signals were corrected for the buffer contribution.

4.2.10 Visible redox titrations

Potentiometric redox titrations of cytochrome PpcE were followed by visible spectroscopy at 15 °C inside an anaerobic glove box (MBraun, Garching, Germany) with oxygen levels kept under 1 ppm, as previously described on section 3.2.4.

Protein solutions (12 μ M) were prepared in 80 mM sodium phosphate with NaCl (250 mM final ionic strength) at pH 7.0, 8.0 and 8.8. As mentioned, a [protein]:[redox mediators] ratio of approximately 10 was found to be optimal to assure good redox equilibrium with the electrode and to avoid specific interactions of mediator molecules with the protein. This was also confirmed by acquiring 1D ^1H NMR spectra of PpcF in the presence and absence of the mixture of redox mediators used in the redox titrations (Figure 4.7). The reduced fraction of PpcF was determined by integrating the area of the α -peak (553 nm) above the line connecting the flanking isosbestic points (545 and 559 nm) to subtract the optical contribution of the redox mediators.

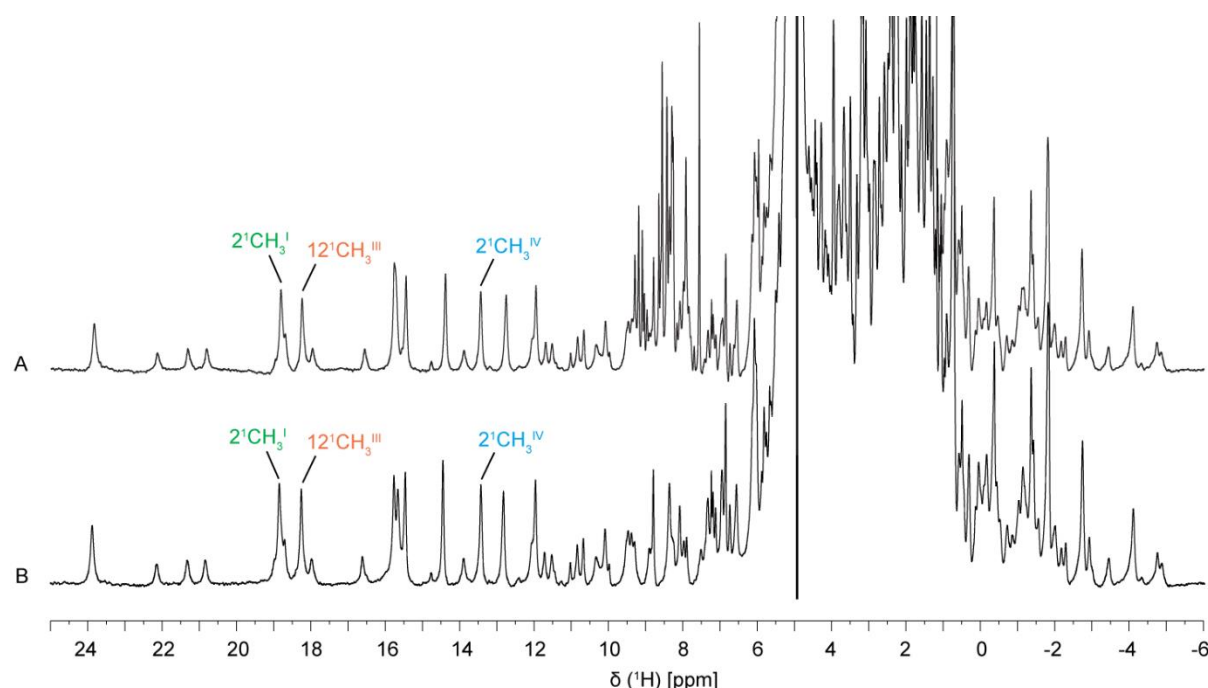


Figure 4.7 - 1D ^1H -NMR spectra of PpcF in the presence (A) and absence (B) of redox mediators obtained at 15 °C and pH 7.5. Protein and redox mediators solution were prepared in 80 mM sodium phosphate with NaCl (250 mM final ionic strength) at the same [protein]:[redox mediators] ratio used in the visible redox titrations (A). The heme methyl signals used to monitor the heme oxidation fractions in the 2D ^1H -EXSY NMR experiments are labeled in both spectra and were unaffected by the presence of the redox mediators.

4.2.11 Thermodynamic model

The thermodynamic model used in the present Thesis was summarized in section 2.3. In the present Thesis, the stepwise oxidation of the hemes in the cytochrome PpcF from *G. metallireducens* was measured by 2D ^1H -EXSY NMR experiments acquired in the pH range 6.0–8.5. The NMR data was fitted simultaneously with data obtained from visible redox titrations carried out at pH 7.0, 8.0 and pH 8.8. The experimental uncertainty of the NMR data was evaluated from the line width of each NMR signal at half height, whereas the visible data points were given an uncertainty of 3% of the total optical signal.

4.2.12 Determination of the axial ligands geometries

The ^{13}C Fermi contact shifts of the heme α -substituents of PpcF at 15 and 25 °C were fitted to a model of the paramagnetic low-spin heme molecular orbitals [19], which is semi-empirically related to the axial ligands geometry. In the oxidized state, each heme group of the cytochrome contains an unpaired electron in the iron d-orbitals that is partially delocalized into the porphyrin π -orbitals. The model applied considers that the ^{13}C nuclei of the α -substituents of the hemes experience Fermi contact shifts proportional to the fraction of occupancy of the unpaired electron in the adjacent carbon, belonging to the conjugated π system. Therefore, the pattern of paramagnetic shifts of these α -substituents can be used to deduce the shape of the frontier molecular orbitals of the porphyrin, which is defined by the orientation of the rhombic perturbation (θ , also labeled as π in the literature [22]) that generates the energy splitting of these orbitals (ΔE). The orientation of the rhombic perturbation agrees with the orientation of the bisector of the acute angle (β) defined by the normal to the planes of the axial ligands, as reported for different bis-histidinyI cytochromes [23]. This orientation is defined relative to the N_C - N_A axis of the porphyrin, with positive angles between N_C - N_B - N_A (Figure 4.17). The energy splitting parameter ΔE has a value similar to the rhombic splitting of the Kramers doublets, that arise from mixing d_{xz} and d_{yz} orbitals of the iron in a cubic crystal field with axial and rhombic distortions. This parameter relates to the acute angle β , defined between the axial ligand planes, via the empirical equation $\Delta E = (5 + \cos 4\theta) \cos \beta$ [24]. This information also allows the placing of the magnetic axes relative to the heme frame, considering that the χ_{zz} axis is normal to the heme plane and that the rhombic perturbation and the χ_{yy} axis are related by a counter-rotation [23, 25, 26]. Nevertheless, several studies with cytochromes possessing hemes with various distortions and with the χ_{zz} axis tilted away from the normal to the heme showed that this approach can also be used in such cases, having found a good correlation between the heme molecular orbitals, the axial ligand orientations, and the magnetic susceptibility tensor [17, 24, 27]. The fits were performed using dedicated macros in Microsoft Excel and the errors of the parameters were assessed by random variation of the input ^{13}C chemical shifts, with a standard deviation of ± 1 ppm. The hyperfine constant was fixed at -36 MHz [24] and average values of chemical shifts, measured

in several cytochromes, were used as diamagnetic reference (12.1, 36.5 and 22.5 ppm for heme methyl, thioether and α -propionates groups, respectively) [17]. Finally, the results obtained with the fits of the ^{13}C chemical shifts were cross-checked by applying the empirical equation $\delta_i \text{ (ppm)} = \cos\beta [38 \sin^2 (\theta_i - \Phi) - 4.1 \cos^2 (\theta_i - \Phi) - 15.9] + 13.8$ [23] to the ^1H chemical shifts of the heme methyl groups. In this equation, β is the acute angle between the heme axial histidine planes, θ_i refers to the position of the i^{th} methyl substituent (average of the methyl protons positions [23]), and Φ is equivalent to the angle between the bisector of the angle β and the N_C - N_A axis of the porphyrin.

4.3 Backbone and side chain resonance assignments of triheme cytochrome PpcF from *Geobacter metallireducens*

4.3.1 Results and discussion

In what concerns the assignment of the backbone and side chains of PpcF cytochrome it was performed in the reduced state, where the combined analysis of 2D ^1H , ^{15}N -HSQC and a series of 3D NMR spectra (3D HNCACB, 3D HN(CO)CACB, 3D HNCA, 3D HN(CO)CA) led to the near-complete assignment of backbone ^{15}N (97%), ^1HN (98%), $^{13}\text{C}_\alpha$ (99%) and for $^{13}\text{C}_\beta$ (98%). Figure 4.8 shows the 2D ^1H , ^{15}N -HSQC NMR spectrum of labeled PpcF with the backbone nitrogen resonances assigned as well as those of the side chains of asparagine (Asn³³), glutamine (Gln²¹) and histidine (His¹⁷, His²⁰, His³¹, His⁴⁷, His⁵⁴, His⁶⁸) residues. To extend the assignment to aliphatic protons such as $^1\text{H}_\alpha$, $^1\text{H}_\beta$, $^1\text{H}_\gamma$, $^1\text{H}_\delta$ and $^1\text{H}_\epsilon$ and aliphatic carbons $^{13}\text{C}_\gamma$, $^{13}\text{C}_\delta$ and $^{13}\text{C}_\epsilon$ we analyzed the 2D ^1H , ^{13}C -HSQC and 3D spectra: HCC(H)-TOCSY and HC(C)H-TOCSY. However, in the case of cytochrome PpcF, it was not possible to unequivocally assign the NH and side chain resonances using these spectra alone, as some signals were too broad and therefore not visible in the acquired spectra. Consequently, 3D-NOESY spectra were crucial to achieve a reliable assignment. The assignment is reported in Table 8.1 (Appendix).

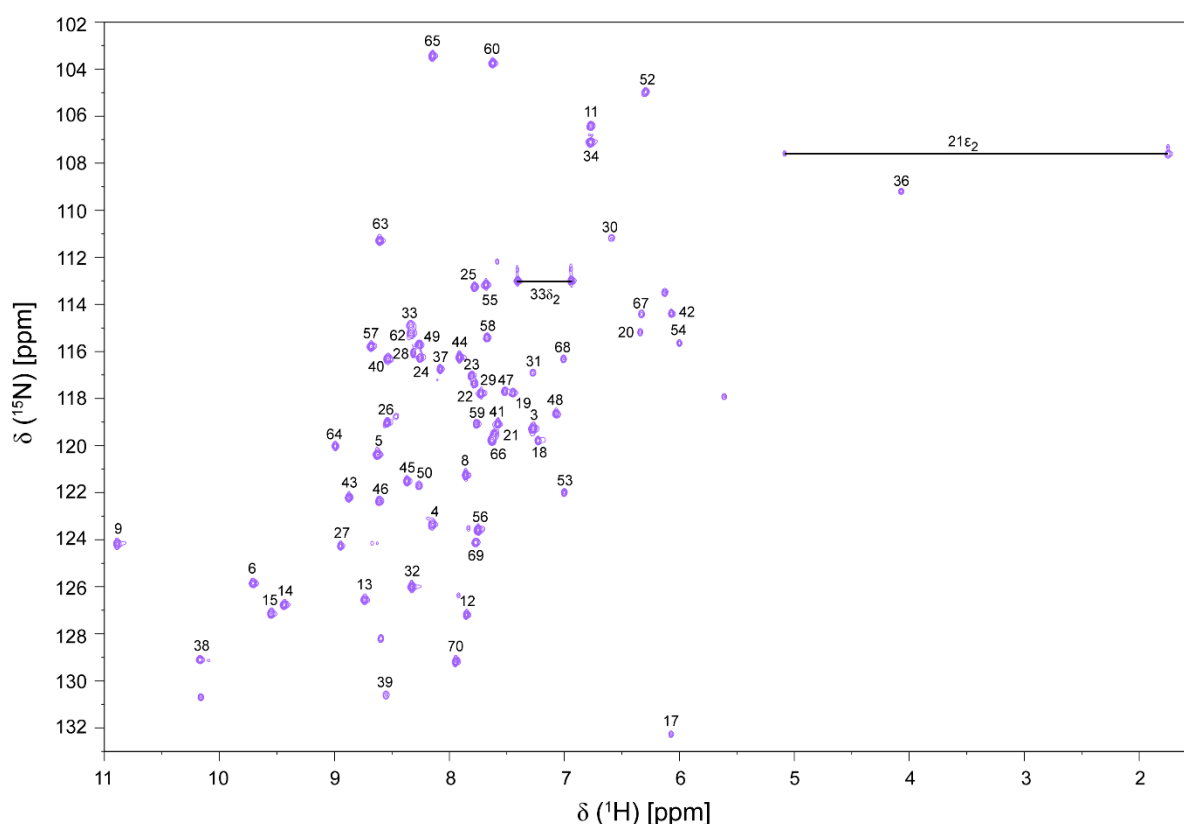


Figure 4.8 - 2D ^1H , ^{15}N -HSQC spectrum of PpcF from *G. metallireducens* in the reduced state at pH 7 and 25 °C. Backbone ^1H - ^{15}N peaks are labeled. The protons associated to the same nitrogen atom (Gln²¹ NH $_{\epsilon 2}$, Asn³³ NH $_{\delta 2}$) are connected by a straight line.

4.4 Thermodynamic properties of triheme cytochrome PpcF from *Geobacter metallireducens*

4.4.1 Results and Discussion

4.4.1.1 PpcF cytochrome stability

The far UV-CD spectra obtained at 25 and 95 °C are indicated in Figure 4.9 at 25 °C, the CD spectrum displays the typical features of a folded protein with high α -helix content, as depicted by the intense negative bands at 206 and 223 nm. The slightly displacement of these two negative bands compared to the typical values observed for α -helix at 208 and 222 nm is probably due to the considerable contribution of the three heme groups in such a small protein. The spectrum also presents a local maximum at 213 nm and a positive ellipticity above 250 nm. Upon heating to 95 °C, small linear changes in ellipticity were observed at 235 nm, except for the signal at 223 nm, which gets about 20 % weaker, and for the local maximum at 213 nm, which redshifts slightly to 216 nm.

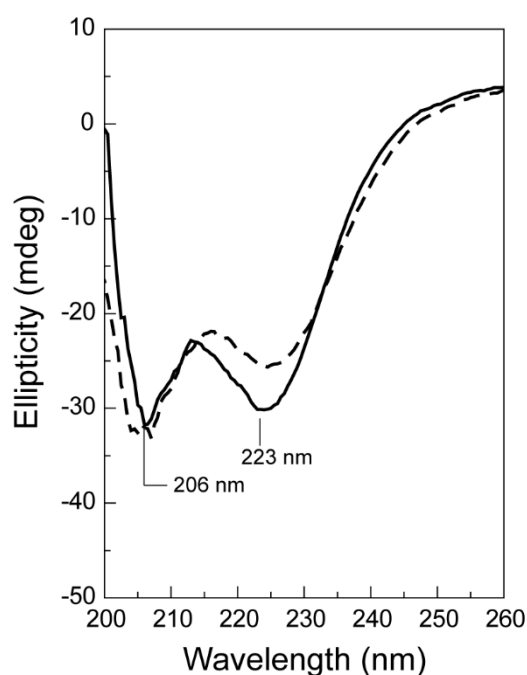


Figure 4.9 - Far UV-CD spectrum of cytochrome PpcF from *G. metallireducens*. Spectrum of the native state at 25 °C is represented by solid line and spectrum upon heating to 95 °C is represented in dashed line. The more intense negative bands, typical of α -helix, are labeled.

Upon re-cooling to 25 °C, the final CD spectrum is essentially equivalent to the initial spectrum, indicating that the protein is very stable. The comparison between the amino acid sequences of the periplasmic triheme cytochromes from *G. metallireducens* and *G. sulfurreducens* suggests that the heme groups in cytochrome PpcF are covalently linked to the polypeptide chain and have bis-histidinyll axial coordination (Figure 1.5). Therefore, it is

expected that the protein shows high stability ($T_m > 85\text{ }^{\circ}\text{C}$), as it was also observed for all PpcA-family cytochromes from *G. sulfurreducens* [4].

4.4.1.2 Thermodynamic parameters

The pH dependence of the heme methyl NMR chemical shifts of PpcF from *G. metallireducens* measured at different oxidation stages (see Figure 4.10 and Table 4.1), together with visible redox titrations data obtained at pH 7.0, 8.0 and 8.8, were fitted to the thermodynamic model summarized in section 2.3 (Figure 4.11).

Table 4.1 - Redox-dependence of the heme methyl proton chemical shifts and heme oxidation fractions of PpcF from *G. metallireducens* (pH 7.5 and 15 °C). The heme methyls $2^1\text{CH}_3^{\text{I}}$, $12^1\text{CH}_3^{\text{III}}$ and $2^1\text{CH}_3^{\text{IV}}$ were chosen to monitor each heme oxidation through the four oxidation stages. The heme oxidation fractions, x_i , in each stage of oxidation, were calculated according to the equation $x_i = (\delta_i - \delta_0) / (\delta_3 - \delta_0)$, where δ_i , δ_0 and δ_3 are the observed chemical shifts of the heme methyl in stage i , 0 (fully reduced) and 3 (fully oxidized), respectively.

Oxidation Stage	Chemical shift (ppm)			x_i			Σx_i
	I	III	IV	I	III	IV	
0	3.42	3.51	3.73	0.00	0.00	0.00	0.00
1	n.d.*	4.38	11.74	0.12*	0.06	0.82	1.00
2	15.96	5.98	12.98	0.81	0.17	0.95	1.93
3	18.85	18.24	13.49	1.00	1.00	1.00	3.00

*The chemical shift of methyl $2^1\text{CH}_3^{\text{I}}$ in the oxidation stage 1 is located underneath the water signal is not observable. Thus, the oxidation fraction of heme I was estimated from the values obtained for the other hemes.

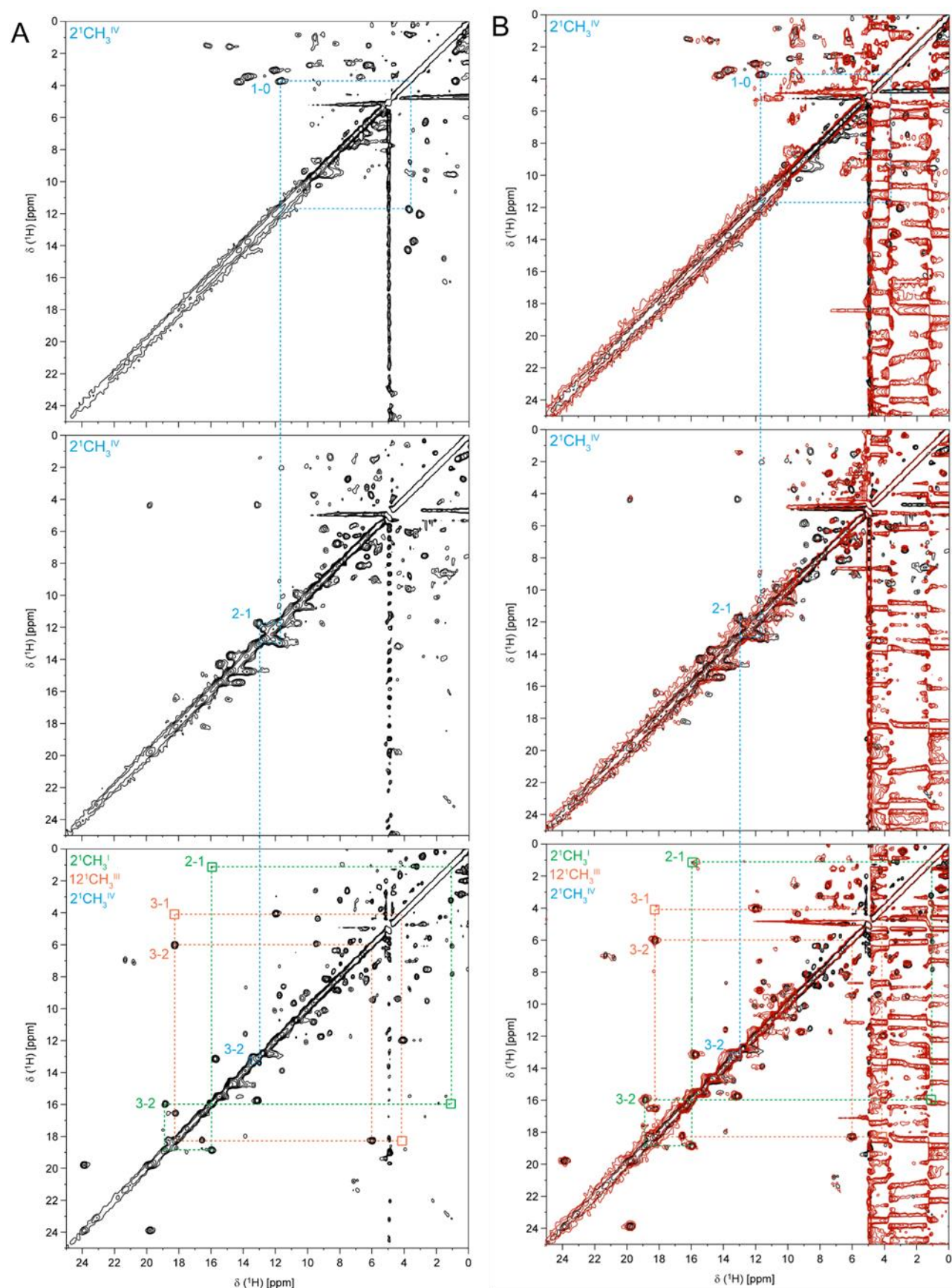


Figure 4.10 – 2D ^1H -EXSY NMR spectrum of PpcF at intermediate stages of oxidation and in the presence of redox mediators. (A) 2D ^1H -EXSY NMR spectrum of cytochrome PpcF obtained at intermediate stages of oxidation (15 °C and pH 7.5). The level of oxidation of the samples increases from the top to the bottom spectrum. Cross-

peaks connecting the chemical shifts of the heme methyl groups in different oxidation stages are indicated by dotted lines. The Roman and Arabic numbers indicate the heme group and the oxidation stage, respectively. The empty boxes correspond to the position of the signals that are only visible at lower contour levels. (B) Overlay of 2D ^1H -EXSY NMR spectra of cytochrome PpcF obtained at intermediate stages of oxidation (15 °C and pH 7.5) in the absence (black contours) and presence (red contours) of redox mediators. The cross-peaks in the two samples are superimposable indicating that the heme oxidation fractions are unaffected by the redox mediators.

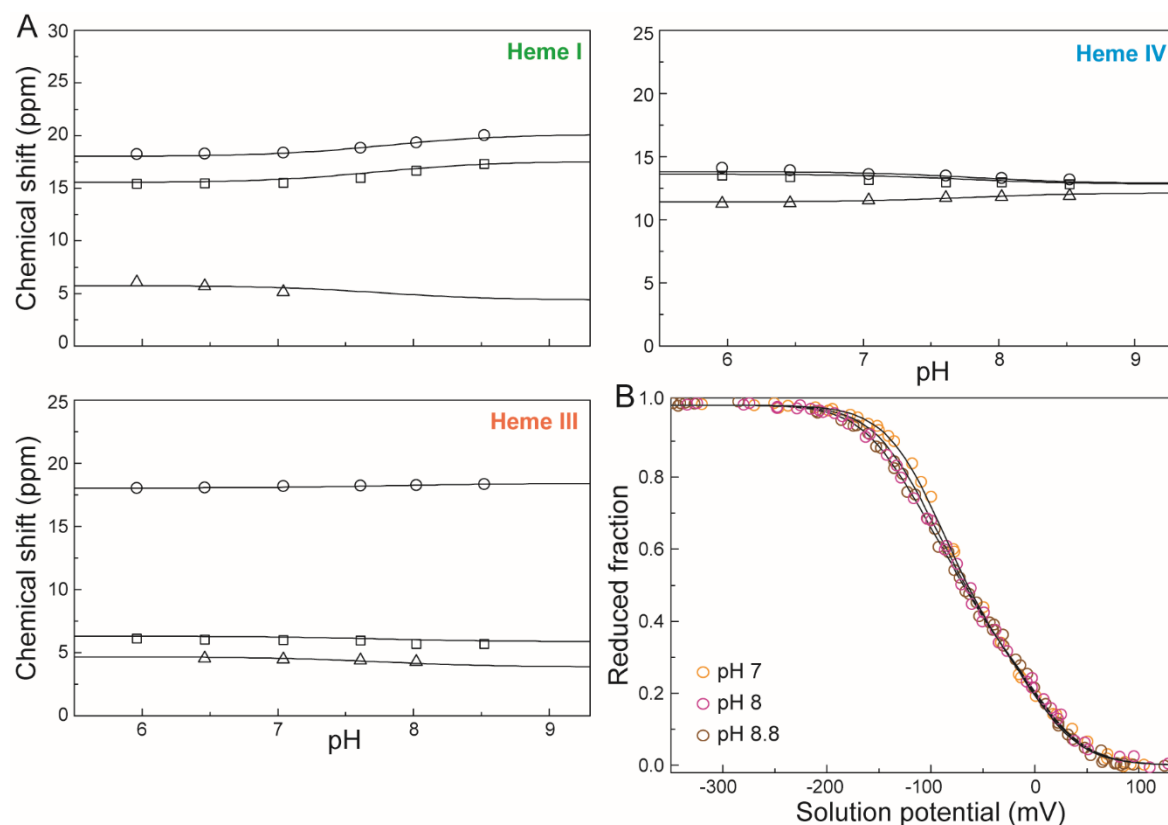


Figure 4.11 - Fitting of the thermodynamic model to the experimental data for PpcF from *G. metallireducens*. The solid lines are the result of the simultaneous fitting of the NMR (A) and visible (B) data. In panel A, the figures show the pH dependence of the heme methyl chemical shifts at oxidation stages 1 (Δ), 2 ($\square$) and 3 ($\circ$). The chemical shifts of the heme methyls in the fully reduced stage (stage 0) are not plotted since they are unaffected by the pH. In panel B, the figure corresponds to the reduced fractions of the cytochrome determined by visible spectroscopy at pH 7, 8 and 8.8 (yellow, pink and brown circles, respectively).

The redox parameters, as well as the macroscopic pK_a values of the redox-Bohr center in the four stages of oxidation, are indicated in Table 4.2 and Table 4.3, respectively. The results show that: (i) the microscopic reduction potential values of the hemes in the reduced and protonated state are negative and different from each other (-65 , -48 and -104 mV for hemes I, III and IV, respectively), (ii) the higher redox interactions are observed for the closest pairs of hemes (29 mV and 16 mV for heme pairs I–III and III–IV, respectively) and (iii) the higher redox-Bohr interaction is observed for heme IV (-29 mV).

Table 4.2 - Energy parameters (meV) for the triheme cytochrome PpcF from *G. metallireducens*. Diagonal terms (in bold) represent the oxidation energies of the three hemes and the deprotonating energy of the redox-Bohr center in the fully reduced and protonated protein. Off-diagonal values are the redox (heme-heme) and redox-Bohr (heme-proton) interaction energies. The standard errors are given in parenthesis. For comparison, the energy parameters obtained for triheme cytochrome PpcA from *G. metallireducens* (*Gm*) and triheme cytochromes from *G. sulfurreducens* (*Gs*) are also indicated.

Energy (meV)				
<i>Gm</i> PpcF	Heme I	Heme III	Heme IV	Redox-Bohr center
Heme I	-65 (5)	29 (3)	-5 (5)	2 (4)
Heme III		-48 (5)	16 (5)	5 (4)
Heme IV			-104 (4)	-29 (5)
Redox-Bohr center				468 (10)
<i>Gm</i> PpcA [28]	Heme I	Heme III	Heme IV	Redox-Bohr center
Heme I	-80 (6)	35 (4)	3 (5)	-22 (6)
Heme III		-70 (7)	37 (7)	-23 (7)
Heme IV			-113 (6)	-49 (6)
Redox-Bohr center				463 (13)
<i>Gs</i> PpcA [29]	Heme I	Heme III	Heme IV	Redox-Bohr center
Heme I	-154 (5)	27 (2)	16 (3)	-32 (4)
Heme III		-138 (5)	41 (3)	-31 (4)
Heme IV			-125 (5)	-58 (4)
Redox-Bohr center				495 (8)
<i>Gs</i> PpcB [29]	Heme I	Heme III	Heme IV	Redox-Bohr center
Heme I	-150 (3)	17 (2)	8 (2)	-16 (4)
Heme III		-166 (3)	32 (2)	-9 (4)
Heme IV			-125 (3)	-38 (4)
Redox-Bohr center				426 (8)
<i>Gs</i> PpcD [29]	Heme I	Heme III	Heme IV	Redox-Bohr center
Heme I	-156 (6)	46 (3)	3 (4)	-28 (6)
Heme III		-139 (6)	14 (4)	-23 (6)
Heme IV			-149 (6)	-53 (6)
Redox-Bohr center				501 (8)
<i>Gs</i> PpcE [29]	Heme I	Heme III	Heme IV	Redox-Bohr center
Heme I	-167 (4)	27 (3)	5 (3)	-12 (4)
Heme III		-175 (4)	22 (3)	2 (4)
Heme IV			-116 (5)	-13 (4)
Redox-Bohr center				445 (10)

Table 4.3 - Macroscopic pK_a values of the redox-Bohr center in cytochrome PpcF from *G. metallireducens*. The values were calculated with the parameters presented in Table 4.2. For comparison, the macroscopic pK_a values obtained for triheme cytochrome PpcA from *G. metallireducens* (*Gm*) and triheme cytochromes from *G. sulfurreducens* (*Gs*) are also indicated.

	Oxidation stage				ΔpK_a
	0	1	2	3	
<i>Gm</i> PpcF	8.2	7.7	7.7	7.7	0.5
<i>Gm</i> PpcA [28]	8.1	7.3	6.9	6.5	1.6
<i>Gs</i> PpcA [29]	8.6	8.0	7.2	6.5	2.1
<i>Gs</i> PpcB [29]	7.4	7.1	6.8	6.3	1.1
<i>Gs</i> PpcD [29]	8.7	8.1	7.4	6.9	1.8
<i>Gs</i> PpcE [29]	7.7	7.6	7.5	7.4	0.3

The analysis of the microscopic redox potential values permitted to establish the order of oxidation of the hemes for the fully reduced and protonated protein, which is IV-I-III. As for PpcA from *G. metallireducens*, the order of oxidation of the hemes in PpcF is quite distinct from those observed for others triheme cytochromes from *Geobacter* species (Figure 4.12A). In fact, only for the two cytochromes from *G. metallireducens*, the heme IV has the lowest redox potential value. In addition, it is also interesting to note that *G. metallireducens*' cytochromes cover a less negative working redox potential range compared to those from *G. sulfurreducens* (Figure 4.12A). There are several factors capable of affecting the reduction potentials in multiheme cytochromes [30]. From these factors, heme solvent accessibility and charge distribution are the most important [31]. Indeed, the high oxidation fraction values of heme IV in the first redox step, clearly indicate that the reduced state of this heme is more destabilized compared to the other two hemes. The absence of Lys⁹ and Lys⁶⁴, which are both conserved in *G. sulfurreducens* cytochromes and located in the vicinity of heme IV, is most likely the reason for the high oxidation fraction values observed for this heme. In fact, the replacement of positively charged lysine residues by noncharged ones in the vicinity of PpcF heme IV is expected to stabilize its oxidized form and thus facilitate its oxidation.

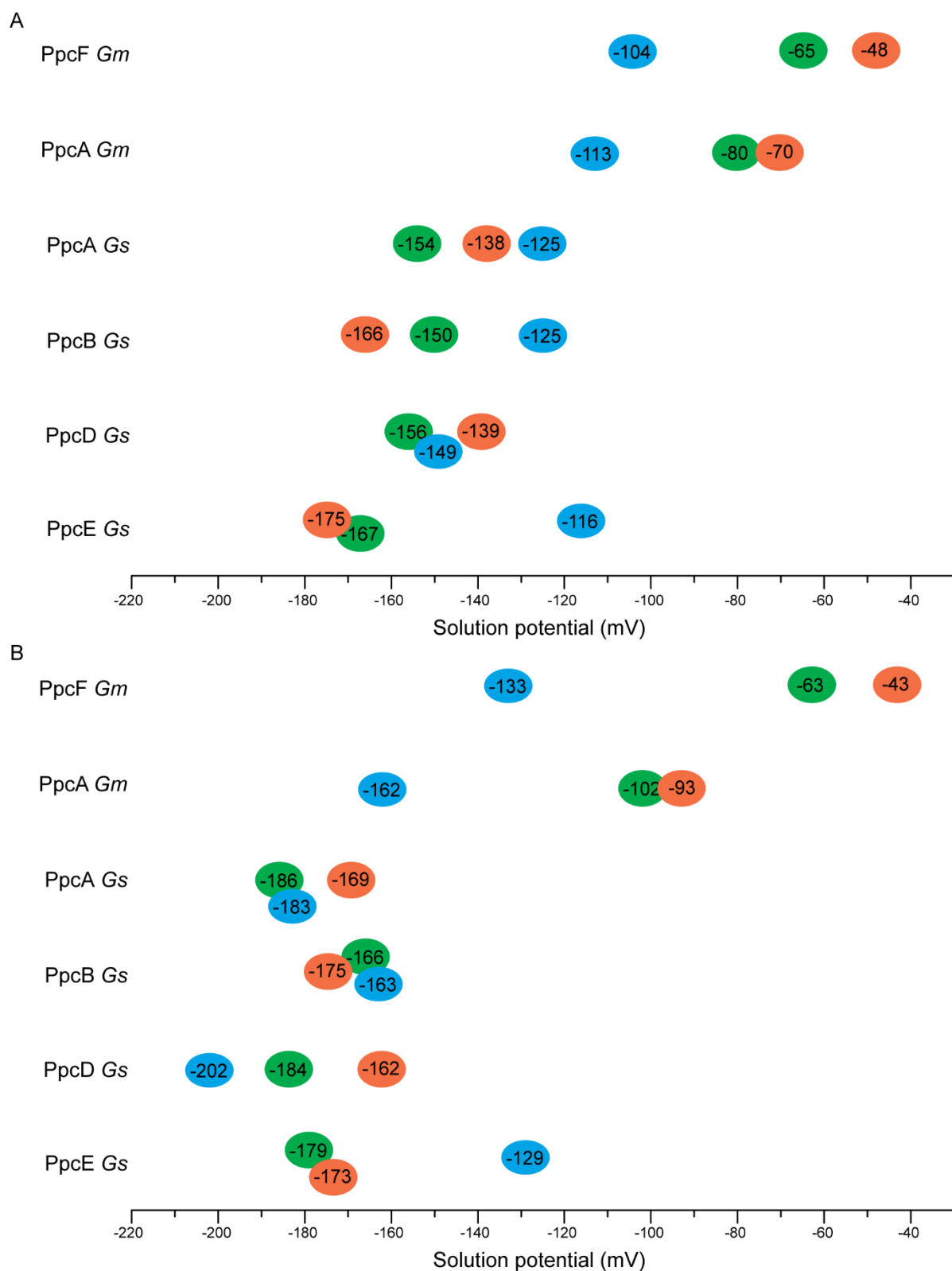


Figure 4.12 - Heme reduction potential values for triheme cytochromes from *G. metallireducens* (*Gm*) and *G. sulfurreducens* (*Gs*) in the reduced protonated (A) and deprotonated (B) forms. In panel A, the reduction potential values were taken directly from Table 4.2. In panel B, the heme reduction potential values were obtained by the sum of the redox-Bohr interactions and the heme reduction potential values in the protonated form (see Table 4.2).

4.4.1.3 Modulation of the redox potential values by heme-heme redox interactions

As mentioned above, the larger redox interactions in PpcF were observed for the closest pair of hemes (I-III and III-IV), a feature also shared with the other triheme cytochromes (Table 4.2). The magnitude of the redox interactions has been shown to correlate with the heme iron-iron distances [32]. Therefore, given the highly structural conservation of the heme core within the triheme cytochromes [29], the observed pattern for the redox interactions is expected. The differences in the values amongst the triheme cytochromes are likely related with small variations in the iron-iron distances and with the nature of the amino acids surrounding the heme groups. Overall, the significant redox interaction values observed for the closest heme pairs indicate that their redox properties are modulated during the oxidation of the proteins. The positive values of the redox interactions indicate that the oxidation of a particular heme delays the oxidation of its neighbor. From a simple electrostatic view of the heme iron charge, the loss of a negative charge caused by the oxidation of one heme is expected to stabilize the reduced form of its neighbor, thus increasing its redox potential value.

4.4.1.4 Modulation of the redox potential values by redox-Bohr interactions

The term redox-Bohr effect reports to the modulation of the redox potential by the pH (for a review see [33]). The redox parameters obtained for cytochrome PpcF also pinpointed important redox-Bohr interactions, particularly for heme IV, thus indicating that its redox potential is also modulated by the solution pH (Table 4.2). This is also clearly observable from the analysis of the first third of the visible redox titration curves obtained at different pH values (Figure 4.11B). The curves are superimposable with the exception of the low solution potential region, which is dominated by the oxidation of heme IV, the first heme to oxidize. Due to the higher redox-Bohr interaction with heme IV (-29 mV) its redox potential decreases with the progressive deprotonation of the redox-Bohr center. In the remaining two thirds of the redox curves, they are undistinguishable, which further corroborates the negligible redox-Bohr interactions obtained for hemes I and III.

As for the redox interactions, from a simple electrostatic view, negative values for the redox-Bohr interactions are expected as the loss of a positive charge in the heme vicinity, upon deprotonation of the redox-Bohr center, stabilizes the oxidized form of the heme group. The effect of the pH modulation on the heme redox potential values is illustrated in Figure 4.12B for the fully reduced and deprotonated protein. The comparison of both panels in Figure 4.12 clearly illustrates that the modulation of the redox properties of the heme groups is quite distinct amongst the triheme cytochromes, thus reflecting the different magnitude of the redox-Bohr interactions. In fact, whereas for some cytochromes the order of oxidation of the

hemes is unaltered in both the protonated and deprotonated forms (PpcA and PpcF from *G. metallireducens*) for others it is significantly affected (triheme cytochromes from *G. sulfurreducens*).

Unlike the redox interactions, which correlate with the heme iron-iron distances, the redox-Bohr interactions are generally more variable. Nevertheless, the highest negative values are consistently observed for heme IV (Table 4.2). This suggests that the redox-Bohr center is located in the vicinity of this heme group. The different magnitudes of the redox-Bohr effect are also reflected on the macroscopic pK_a values (Table 4.3), from which it is clear that PpcF from *G. metallireducens* shows one of the smallest values ($\Delta pK_a = 0.5$). This can be further confirmed by the observation of the visible redox titration curves (Figure 4.11B) for which the macroscopic apparent midpoint reduction potential values are very similar: -65, -67 and -69 mV at pH 7.0, 8.0 and 8.8, respectively. A larger redox-Bohr effect indicates that the cytochrome is able to couple electron/proton transfer, which would be functionally relevant if observed within a physiological pH range (see next section).

4.4.1.5 Heme oxidation profiles in the physiological pH range

As discussed, the apparent heme midpoint reduction potential values (e_{app}) of cytochrome PpcF are tuned by redox and redox-Bohr interactions. Thus, the thermodynamic parameters of PpcF listed in Table 4.2 can be used to determine the e_{app} values of the hemes at physiological pH (Figure 4.13). The results obtained clearly showed that the order of oxidation of the hemes (IV-I-III) is pH independent. As noted for the fully reduced and protonated protein, by having the lowest reduction potential value, heme IV is the first heme to oxidize (Figure 4.12A). Due to the relatively high pK_a value of the redox-Bohr center (Table 4.3), the difference between the first heme to oxidize (heme IV) and the second one (heme I) is even more notorious at high pH (Figure 4.13). On the other hand, the pH has little effect on the e_{app} values of the other two hemes due to their small redox-Bohr interactions (2 and 5 mV for hemes I and III, respectively – see Table 4.2).

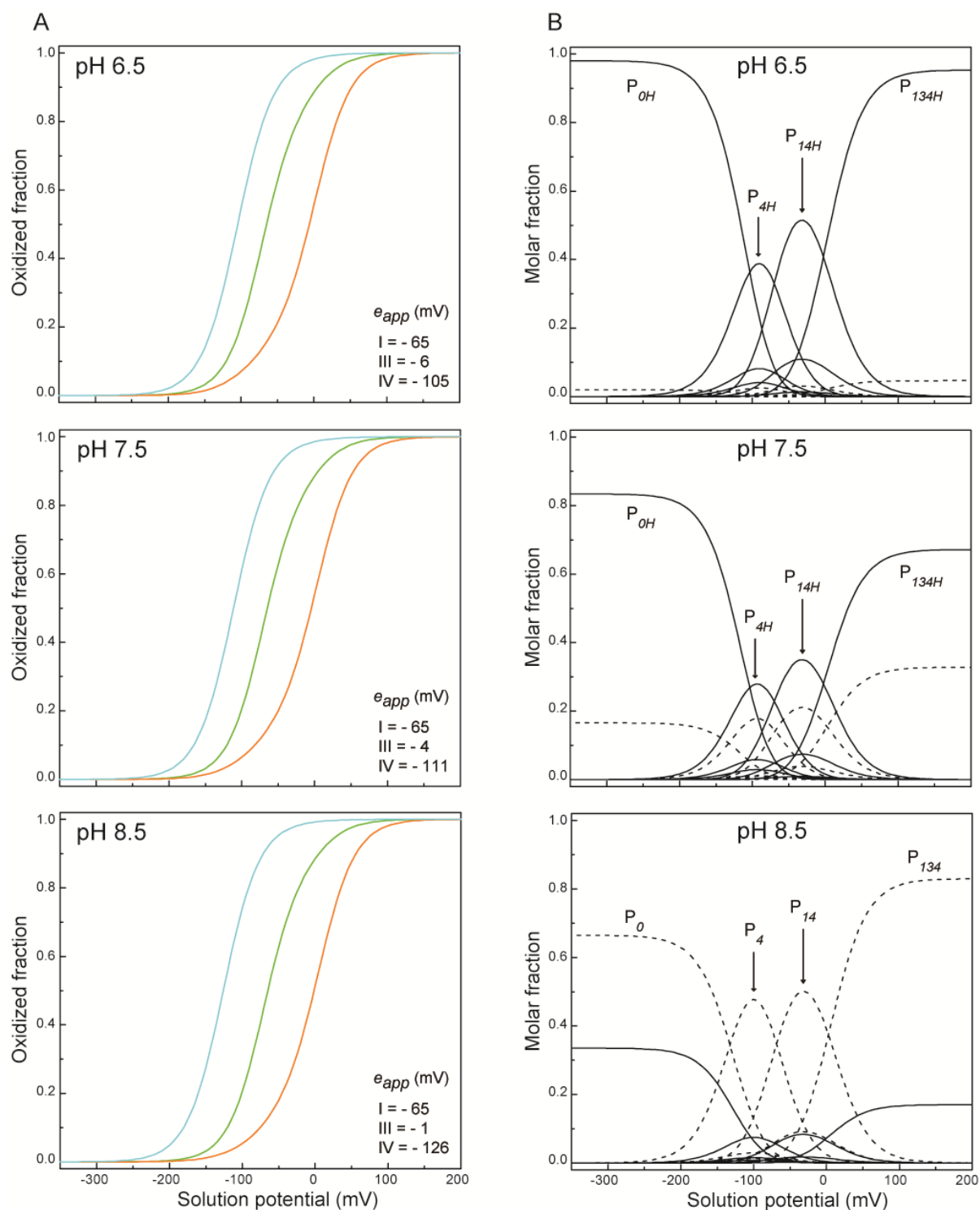


Figure 4.13 - Redox and pH-dependence of the individual heme oxidation fractions (A) and molar fractions of the 16 microstates (B) of PpcF from *G. metallireducens*. The curves were calculated as a function of the solution reduction potential (relative to NHE) using the parameters listed in Table 4.2. In the left panels, the hemes I, III and IV curves are represented in green, orange and blue, respectively. The midpoint reduction potentials of the hemes (e_{app}) are indicated. In the right panels, the solid and dashed lines indicate the protonated and deprotonated microstates, respectively (see Figure 2.2). For clarity, only the dominant microstates are labeled.

The pK_a value of the redox-Bohr center and the small redox interaction between hemes I and IV explain the similar values observed for their midpoint redox potential values at pH 6.5 compared to their values in the fully reduced and protonated protein (Figure 4.12A and Figure 4.13A). In fact, during the oxidation of heme IV and because of the small redox interaction between hemes I and IV (-5 mV), the reduction potential value of heme I is essentially unaffected. On the other hand, a significant difference on the midpoint redox potential of heme III is observed in the pH range 6.5 to 8.5 (-6 to -1 mV) compared to its value in the fully protonated and reduced protein (-48 mV) (Figure 4.12A and Figure 4.13A). This difference is explained by the redox interactions between heme III and hemes I and IV (Table 4.2). In fact, since hemes I and IV oxidize clearly before heme III, their redox interactions (29 and 16 mV for I-III and III-IV heme pairs, respectively) increase the redox potential of heme III to higher reduction potential values throughout the protein oxidation. As a result of the established redox and redox-Bohr interactions' network, the midpoint redox potential of the three hemes in PpcF are considerably separated in the entire pH range (Figure 4.13A) and therefore their order of oxidation is pH-independent.

The same oxidation profile was observed for PpcA from *G. metallireducens*, which also differs considerably from the ones observed for the periplasmic triheme cytochromes from *G. sulfurreducens* (Table 4.4). The strong modulation by the pH of the hemes' oxidation profiles, particularly in the case of PpcA and PpcD from *G. sulfurreducens*, is explained by the smaller separation of the heme's reduction potential values and by the non-negligible value of the redox-Bohr interactions (Table 4.2).

Table 4.4 - Comparison of the heme microscopic redox potentials (e_{app}) of triheme cytochromes from *G. metallireducens* (*Gm*) and *G. sulfurreducens* (*Gs*) at pH 6.5, 7.5 and 8.5. The heme microscopic redox potentials were retrieved from the individual heme oxidation fraction curves, which were calculated as a function of the solution reduction potential (relative to NHE), using the parameters listed in Table 4.2.

		e_{app} (mV)		
		pH 6.5	pH 7.5	pH 8.5
<i>Gm</i> PpcF	Heme I	-65	-65	-65
	Heme III	-6	-4	-1
	Heme IV	-105	-111	-126
<i>Gm</i> PpcA [28]	Heme I	-77	-88	-94
	Heme III	-12	-22	-25
	Heme IV	-114	-132	-153
<i>Gs</i> PpcA [29]	Heme I	-145	-152	-166
	Heme III	-101	-108	-112
	Heme IV	-100	-126	-153
<i>Gs</i> PpcB [29]	Heme I	-142	-150	-154
	Heme III	-156	-155	-154
	Heme IV	-107	-130	-138
<i>Gs</i> PpcD [29]	Heme I	-147	-156	-169
	Heme III	-92	-102	-107
	Heme IV	-147	-162	-183
<i>Gs</i> PpcE [29]	Heme I	-152	-158	-164
	Heme III	-162	-158	-153
	Heme IV	-94	-100	-105

The conserved order of oxidation of the hemes and the significant difference between the reduction potential values in cytochrome PpcF from *G. metallireducens* suggest that the microstate with heme IV oxidized will dominate the first oxidation stage (S1), followed by the microstate with both hemes IV and I oxidized in the second oxidation stage (S2). To confirm this, and to elucidate the functional mechanism of PpcF, the fractional contributions of the 16 possible microstates were determined (see Figure 4.13B).

4.4.1.6 Functional mechanism of PpcF at physiological pH

To further rationalize the effect of the redox interactions' network in the functional mechanism of PpcF, the fractional contributions of the 16 microstates were determined in the pH range 6.5–8.5 (Figure 4.13B). As expected, from the analysis of the heme oxidation patterns (Figure 4.13A), particularly by the considerable separation between the heme reduction potential values, all the oxidation stages are dominated by a single microstate. In fact, a well-

defined preferential electron transfer pathway is established: $P_{0H} \rightarrow P_{4H} \rightarrow P_{14H} \rightarrow P_{134H}$ (pH 6.5 and 7.5) or $P_0 \rightarrow P_4 \rightarrow P_{14} \rightarrow P_{134}$ (pH 8.5). However, the dominant microstates are all protonated (pH 6.5 and 7.5) or deprotonated (pH 8.5). In fact, due to the small redox-Bohr effect ($\Delta pK_a = 0.5$ – see Table 4.3) the protonation/deprotonation of the redox-Bohr center coupled to electron transfer will be only observable in a narrow pH range within the pK_a^{red} (8.2) and pK_a^{ox} (7.7) values. Consequently, it is clear that PpcF is unable to perform a concerted e^-/H^+ transfer mechanism, unlike what was observed for PpcA from *G. metallireducens* and PpcA and PpcD from *G. sulfurreducens* at pH 7 (Table 4.5). The three latter cytochromes displayed a preferential e^-/H^+ transfer mechanism at physiological pH because of their larger redox-Bohr effect and range covered by the pK_a values of the redox-Bohr centers (Table 4.3). Compared to the two triheme cytochromes from *G. sulfurreducens* that also show a very small redox-Bohr effect (PpcB and PpcE), PpcF shows a well-established and conserved order of oxidation of the hemes in the entire pH range, which is also independent of the protonation level of the redox-Bohr center (Table 4.3 and Table 4.5).

Table 4.5 - Order of oxidation of the hemes and preferential transfer pathways of triheme cytochromes from *G. metallireducens* (*Gm*) and *G. sulfurreducens* (*Gs*). The pathways were defined by calculating the redox dependence of the molar fractions of the 16 microstates of each cytochrome at physiological pH, using the thermodynamic parameters listed in Table 4.2.

Cytochrome	Order of oxidation of the hemes	Preferential pathways	e^-/H^+ coupled transfer
<i>Gm</i> PpcF	IV – I – III	$P_{0H} \rightarrow P_{4H} \rightarrow P_{14H} \rightarrow P_{134H}$	No
<i>Gm</i> PpcA [28]	IV – I – III	$P_{0H} \rightarrow P_{4H} \rightarrow P_{14} \rightarrow P_{134}$	Yes
<i>Gs</i> PpcA [29]	I – IV – III	$P_{0H} \rightarrow P_{1H} \rightarrow P_{14} \rightarrow P_{134}$	Yes
<i>Gs</i> PpcB [29]	(III,I) – IV	Not defined	No
<i>Gs</i> PpcD [29]	IV – I – III	$P_{0H} \rightarrow P_{14} \rightarrow P_{134}$	Yes
<i>Gs</i> PpcE [29]	(III,I) – IV	Not defined	No

The data obtained shows that PpcF is designed to interact with the physiological electron acceptor and donor via hemes III and IV, respectively. As a first electron is taken by the acceptor, heme III can readily be re-reduced by the neighboring heme I, a step that is thermodynamically favorable given the smaller reduction potential of this heme. Heme I can be then re-reduced by heme IV and thus assure a continuous re-reduction of heme III and electron transfer directionality to the acceptor (Figure 4.14). Considering the large separation of the heme reduction potential values of PpcF and the redox order of the hemes, it is unlikely that its functional mechanism is affected by the presence of nitrate. In fact, the study of the ionic dependence of the redox properties of a PpcF's homologue protein, PpcA from *G. sulfurreducens*, in the range 0.02 to 1 M [34] and also in the presence of a high concentration of small and negatively charged electron acceptors [35], as it is the case of nitrate, showed that

the redox properties are marginally affected and, more importantly, that the functional mechanism is conserved, despite the higher similarity between the heme reduction potentials in PpcA from *G. sulfurreducens* (Table 4.4).

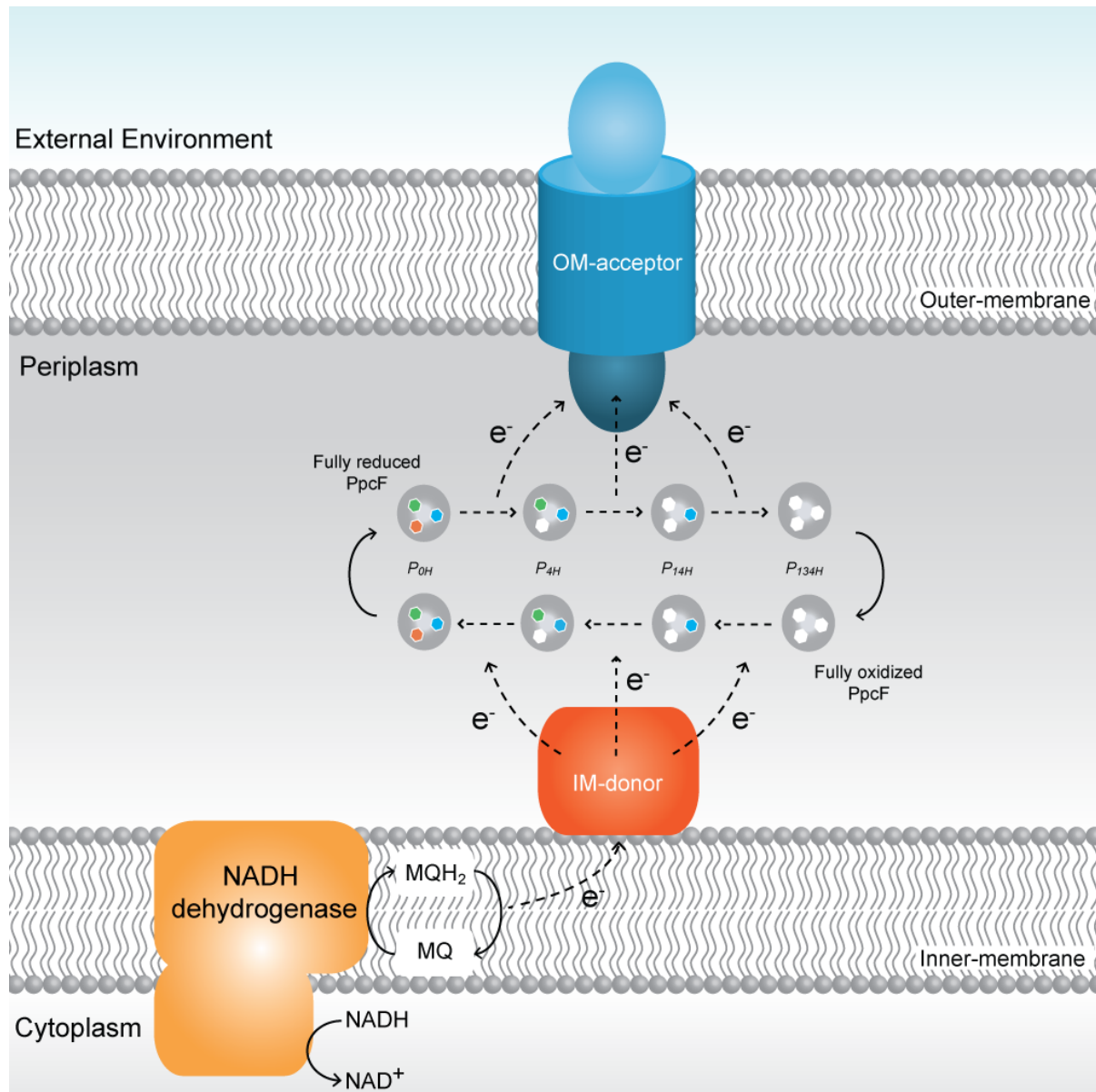


Figure 4.14 - Proposed model for the extracellular electron transfer pathways via PpcF in *G. metallireducens*. The black arrows represent the proposed electron transfer pathways. The NADH dehydrogenase (light orange) catalyzes the oxidation of NADH to NAD⁺ and the resulting electrons are transferred to the menaquinol (MQH₂)/menaquinone (MQ) pool. The electrons flow from this pool to a specific inner-membrane (IM) cytochrome (dark orange). The periplasmic PpcF cytochrome (gray circle) mediates the electron transfer between an IM-associated cytochrome and an outer-membrane (OM) porin-cytochrome complex (blue). The heme groups in PpcF are labeled green, orange and blue for hemes I, III and IV, respectively.

4.5 Molecular geometries of the heme axial ligands from the triheme cytochrome PpcF from *Geobacter metallireducens*

4.5.1 Results and Discussion

4.5.1.1 NMR spectral features of the triheme cytochrome PpcF

The 1D ^1H NMR spectral features of the triheme cytochrome PpcF are typical of low-spin heme groups [36], since the NMR resonances of PpcF cover the spectral regions of -5 to 25 ppm and -2 to 11 ppm in the oxidized and reduced forms, respectively (Figure 4.15). Therefore, the 1D ^1H NMR spectra of PpcF at different oxidation states are quite different. On one hand, in the reduced state, the chemical shifts of the heme substituents are dominated by the ring-current effects caused by the circular movement of electrons around the pyrrole rings, which generates local magnetic fields that determine the regions where the heme proton resonances are observed. On the other hand, in the oxidized state, in addition to the ring-current effects, there are both intrinsic (from its own heme) and extrinsic (from neighboring hemes) paramagnetic contributions, caused by the presence of unpaired electrons, which are responsible for the wider dispersion of the heme substituent chemical shifts. Indeed, and in contrast with the reduced state, in the oxidized form the same type of signals is differently affected by the paramagnetic effects, shows different levels of broadness, and is differently spread over the entire NMR spectral width. This is particularly notable for the heme methyl proton signals that shift from crowded regions in the fully reduced spectrum (2.5 – 5 ppm) to relatively empty regions in the fully oxidized spectrum (from 0 to 25 ppm, Figure 4.15). The heme α -substituent signals of PpcF have been previously reported for the fully reduced state [8] and, in the present Thesis, were obtained for the oxidized form (Table 4.6).

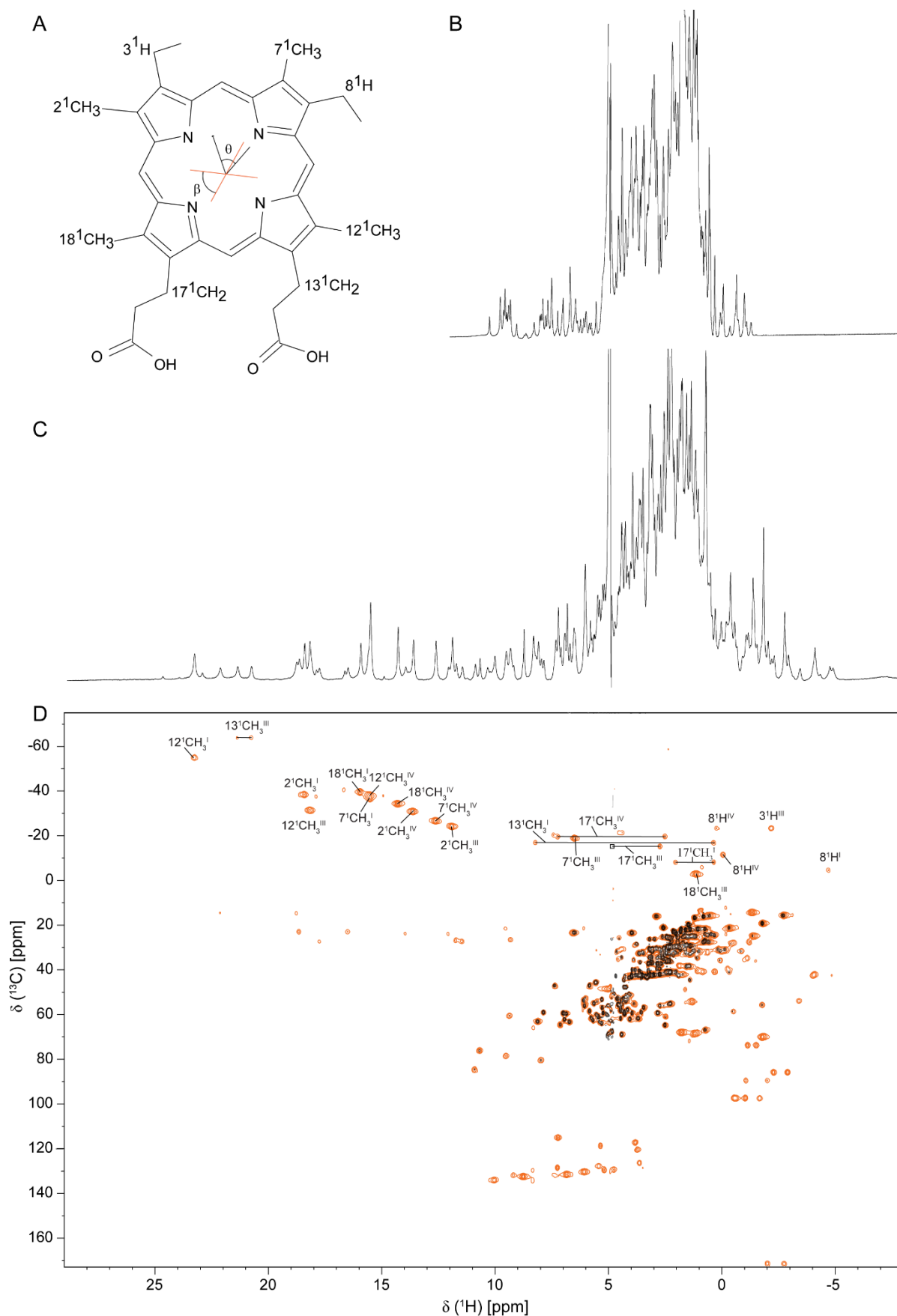


Figure 4.15 - NMR spectral features of PpcF from *G. metallireducens*, at pH 7 and 25 °C. (A) The heme *c* diagram shows the heme pyrrole rings labeled (green letters) according to the Brookhaven Protein Data Bank standard atom nomenclature for metal-substituted protohemes. The β and θ parameters are shown, with the black arrow

representing the orientation of the rhombic perturbation and the black line indicating the N_C-N_A direction. The two orange lines represent the projection of the axial histidine planes on the heme. The heme substituents are labeled according to the IUPAC-IUB nomenclature for tetrapyrroles [14], in both the spectra (panel D) and the heme *c* diagram. (B) 1D ¹H NMR spectrum of the reduced and (C) oxidized cytochrome. (D) Superimposed 2D ¹H,¹³C-HSQC spectra of labeled (black contours) and unlabeled (orange contours) samples of oxidized PpcF. The peaks of the protons connected to the same carbon atoms (CH₂ groups) are linked by a straight line (see detailed assignment on Table 4.6). Roman numerals indicate the hemes in their order of attachment to the CXXCH motif in the polypeptide chain. The PpcF hemes are numbered I, III and IV.

Table 4.6 - ¹H and ¹³C chemical shifts (ppm) of the α -substituent of the three hemes of PpcF from *G. metallireducens* at 15 and 25 °C (pH 7 and 250 mM ionic strength).

T (°C)	Group	Heme I		Heme III		Heme IV	
15		¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
	2 ¹	-38.43	18.42	-24.21	11.91	-30.89	13.63
	3 ¹	-	-	-23.36	-2.21	-11.52	-0.07
	7 ¹	-18.93	6.49	-36.50	15.51	-26.71	12.63
	8 ¹	-4.65	-4.74	-	-	-23.39	0.21
	12 ¹	-54.98	23.28	-31.42	18.19	-37.74	15.53
			8.22		21.39		*
	13 ¹	-16.91	0.34	-63.80	20.76	-21.3	4.38
			2.03		4.91		7.23
	17 ¹	-8.19	0.33	-15.23	2.71	-19.66	2.49
	18 ¹	-39.64	15.96	-2.91	1.12	-34.38	14.30
25							
	2 ¹	-36.26	17.97	-22.88	11.85	-29.50	13.56
	3 ¹	-	-	-21.02	-1.73	-9.79	0.28
	7 ¹	-18.08	6.86	-36.36	15.37	-25.13	12.40
	8 ¹	-3.31	-4.16	-	-	-20.84	0.53
	12 ¹	-52.41	22.70	-30.04	17.93	-35.57	15.50
			8.08		21.21		4.86
	13 ¹	-15.18	0.70	-60.53	20.10	-19.95	4.50
			2.48		4.98		7.44
	17 ¹	-7.24	0.94	-13.69	3.09	-17.90	2.72
	18 ¹	-38.30	16.08	-2.84	1.71	-32.96	14.22

* At this temperature, this resonance is located underneath the water signal (4.93 ppm) and, therefore, this heme α -propionate was not included in the determination of the axial ligand's geometry.

4.5.1.2 NMR resonance assignment of the heme α substituents

Using the assignment strategy previously described for the heme α substituents (4.2.8.4), the 2D ^1H -EXSY NMR spectra were used to follow the assignment of signals through different oxidation stages. In the case of PpcF from *G. metallireducens*, this procedure enabled the assignment of nine heme methyls to their final position: $2^1\text{CH}_3^{\text{I}}$, $12^1\text{CH}_3^{\text{I}}$ and 18^1CH_3 from heme I; 2^1CH_3 and 12^1CH_3 from heme III and the four heme methyls from heme IV. The remaining three heme methyl signals ($7^1\text{CH}_3^{\text{I}}$, $7^1\text{CH}_3^{\text{III}}$ and $18^1\text{CH}_3^{\text{III}}$) have small paramagnetic contributions and/or appear in crowded regions of the ^1H NMR spectra (Figure 4.15), making it difficult to assign via 2D ^1H -EXSY NMR experiments. Using the strategy previously described in section 4.2.8.4 the combined use of 2D ^1H , ^{13}C -HSQC, 2D ^1H -NOESY and 2D ^1H -TOCSY experiments in the fully oxidized state allowed the assignment of the remaining heme α -substituents: the three unassigned heme methyls, the thioether methines 3^1H and 8^1H , as well as the α -propionates 13^1CH_2 and 17^1CH_2 .

With resource to 2D ^1H , ^{13}C -HSQC NMR spectra recorded for an unlabeled sample and for a ^{13}C labeled sample exclusively in its polypeptide chain and the analysis of the intraheme TOCSY and NOESY connectivities (following the strategy described in section 4.2.8.4) the specific assignment of these signals to each heme group was obtained. These procedures allowed the assignment of 93% (28 out of 30) of the heme α -substituents. The small fraction of unassigned peaks corresponds to signals located in crowded spectral regions that could not be confidently assigned.

4.5.1.3 Geometry and magnetic properties of the axial ligands of PpcF

The conceptual model presented for the heme molecular orbitals is only valid for low-spin paramagnetic hemes [19]. As mentioned above, the 1D ^1H NMR spectral features of PpcF suggested that the cytochrome is low-spin. However, an independent test of its spin properties was performed in this Thesis by studying the temperature dependence of the heme methyl substituents in the oxidized state (Figure 4.16). The analysis of this figure shows that the signals follow Curie law behavior, as their chemical shifts decrease with increasing temperature [37]. The linear dependence of the measured chemical shifts with temperature further confirms the low-spin character of the three heme groups (Figure 4.16) and, therefore, the proper conditions for the application of the model of the heme molecular orbitals.

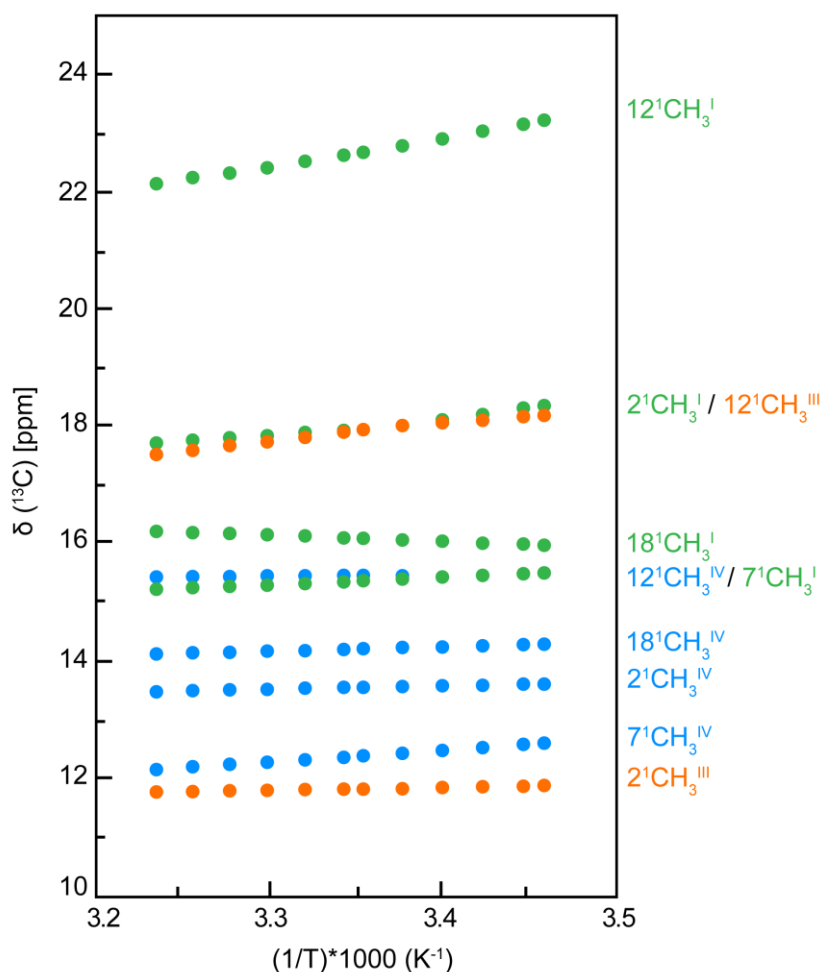


Figure 4.16 - Temperature dependence of PpcF heme methyl signals in the oxidized state, at pH 7 and 250 mM ionic strength. The heme methyl signals (in green, orange and blue for hemes I, III and IV, respectively) of PpcF follow Curie law behavior. The chemical shifts of the heme methyls located at higher field (7^1CH_3^1 and $18^1\text{CH}_3^{\text{III}}$) could not be followed throughout the temperature titration (due to the location of their resonances, see text) and, therefore, are not represented.

The calculation of the orientations of the heme axial histidine ring planes was performed using the ^{13}C NMR paramagnetic shifts of the heme α -substituents at two different temperatures (15 and 25 °C) and the results are reported in Figure 4.17 and Table 4.7. The quality of the fittings obtained (Table 4.7), together with the comparison between the experimental and calculated shifts (Figure 4.17 and Table 4.8), clearly show that the experimental data are well described by the model. The experimental and calculated ^{13}C Fermi contact shifts are also shown as scatter plots in Figure 4.18. The observed deviations occur due to additional effects on chemical shift such as pseudocontact interactions, but mainly because the heme symmetry is lowered by its substituents and deviations from planarity. However, the overall pattern of shifts, which reflects the axial ligand geometry (shown in Figure 4.17) remains clear and the resulting uncertainties may be gauged from Table 4.7.

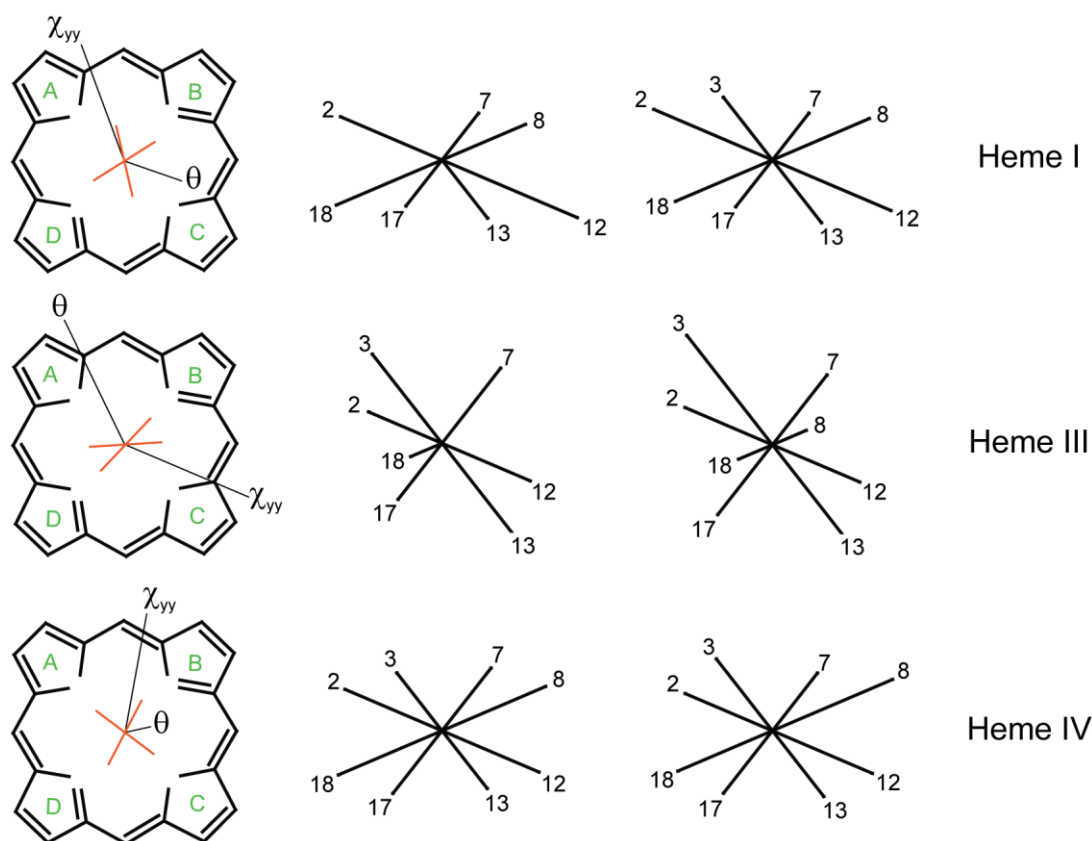


Figure 4.17 - Orientation of the heme axial ligands, and experimental and calculated shifts of the hemes α -substituents of PpcF from *G. metallireducens*. The orientations of the axial histidine planes determined in this Thesis are represented in the first column (in orange), together with the orientation of the rhombic perturbation (θ) and the χ_{yy} magnetic axis for each heme. The length of the line corresponding to the rhombic perturbation is proportional to the magnitude of the orbital energy splitting between the frontier molecular orbitals (ΔE). The last two columns represent the experimental and calculated ^{13}C NMR paramagnetic shifts of the heme α -substituents at 25 °C, respectively. Each line is oriented from the center of the heme to the respective carbon, with length proportional to the paramagnetic shift. The heme pyrrole rings (green letters) and α -substituents are identified as in Figure 4.15. The heme α -substituents are identified according to the IUPAC-IUB nomenclature [14]. The axes are defined relative to the Nc-N_A vector, with a positive angle for a rotation in the counterclockwise direction.

Table 4.7 - Molecular orbital parameters obtained by fitting of the ^{13}C Fermi contact shifts of the heme α -substituents of PpcF from *G. metallireducens*. The standard errors associated with the molecular orbital parameters were calculated assuming that the chemical shifts have an experimental uncertainty of ± 1 ppm. The parameter β was calculated from the energy splitting of the molecular orbitals using an empirical equation (see section 4.2.12). The molecular orbital parameters of PpcA from *G. metallireducens* (*Gm*) and for the PpcA-family cytochromes from *G. sulfurreducens* (*Gs*) are also presented, for comparison.

Protein	Parameters	Heme I	Heme III	Heme IV
PpcF <i>Gm</i>	θ ($^\circ$)	-64.7 ± 0.5	69.2 ± 0.2	-32.9 ± 1.2
	ΔE (J mol $^{-1}$)	1576 ± 24	3745 ± 46	717 ± 23
	β ($^\circ$)	70.8 ± 0.3	42.9 ± 0.8	80.5 ± 0.3
PpcA <i>Gm</i> [38]	θ ($^\circ$)	-67	66	-57
	ΔE (J mol $^{-1}$)	2034	3615	582
	β ($^\circ$)	66	43	82
PpcA <i>Gs</i> [39]	θ ($^\circ$)	-70	67	-63
	ΔE (J mol $^{-1}$)	1350	3540	670
	β ($^\circ$)	74	42	84
PpcB <i>Gs</i> [40]	θ ($^\circ$)	-57	70	-63
	ΔE (J mol $^{-1}$)	640	3540	890
	β ($^\circ$)	81	47	79
PpcC <i>Gs</i> [40]	θ ($^\circ$)	-62	62	-78
	ΔE (J mol $^{-1}$)	1430	2430	2090
	β ($^\circ$)	72	59	69
PpcD <i>Gs</i> [40]	θ ($^\circ$)	-44	69	-21
	ΔE (J mol $^{-1}$)	4240	4170	520
	β ($^\circ$)	0	35	84
PpcE <i>Gs</i> [40]	θ ($^\circ$)	-27	74	-65
	ΔE (J mol $^{-1}$)	840	4180	810
	β ($^\circ$)	80	40	80

Table 4.8 - Experimental and calculated ^{13}C Fermi contact shifts of the heme α -substituents of PpcF, at pH 7 (15 and 25 °C). Both the experimental and calculated Fermi contact shifts were calculated by subtracting the respective diamagnetic references (12.1, 36.5 and 22.5 ppm for heme methyls, thioethers and α -propionates, respectively) [17].

	Group	Experimental shifts (ppm)		Calculated shifts (ppm)	
		15 °C	25 °C	15 °C	25 °C
Heme I	2¹	-50.5	-48.4	-58.9	-56.5
	3¹	-	-	-41.8	-40.5
	7¹	-31.0	-30.2	-30.8	-30.2
	8¹	-41.2	-39.8	-47.9	-46.2
	12¹	-67.1	-64.5	-58.9	-56.5
	13¹	-39.4	-37.7	-41.8	-40.5
	17¹	-30.7	-29.7	-30.8	-30.2
	18¹	-51.7	-50.4	-47.9	-46.2
Heme III	2¹	-36.3	-35.0	-43.1	-41.7
	3¹	-59.9	-57.5	-73.8	-70.7
	7¹	-48.6	-48.5	-46.7	-45.1
	8¹	-	-	-16.0	-16.1
	12¹	-43.5	-42.1	-43.1	-41.7
	13¹	-86.3	-83.0	-73.8	-70.7
	17¹	-37.7	-36.2	-46.7	-45.1
	18¹	-15.0	-14.9	-16.0	-16.1
Heme IV	2¹	-43.0	-41.6	-48.0	-46.3
	3¹	-48.0	-46.3	-38.4	-37.3
	7¹	-38.8	-37.2	-41.8	-40.5
	8¹	-59.9	-57.3	-51.4	-49.4
	12¹	-49.8	-47.7	-48.0	-46.3
	13¹	-	-42.5	-38.4	-37.3
	17¹	-42.2	-40.4	-41.8	-40.5
	18¹	-46.5	-45.1	-51.4	-49.4

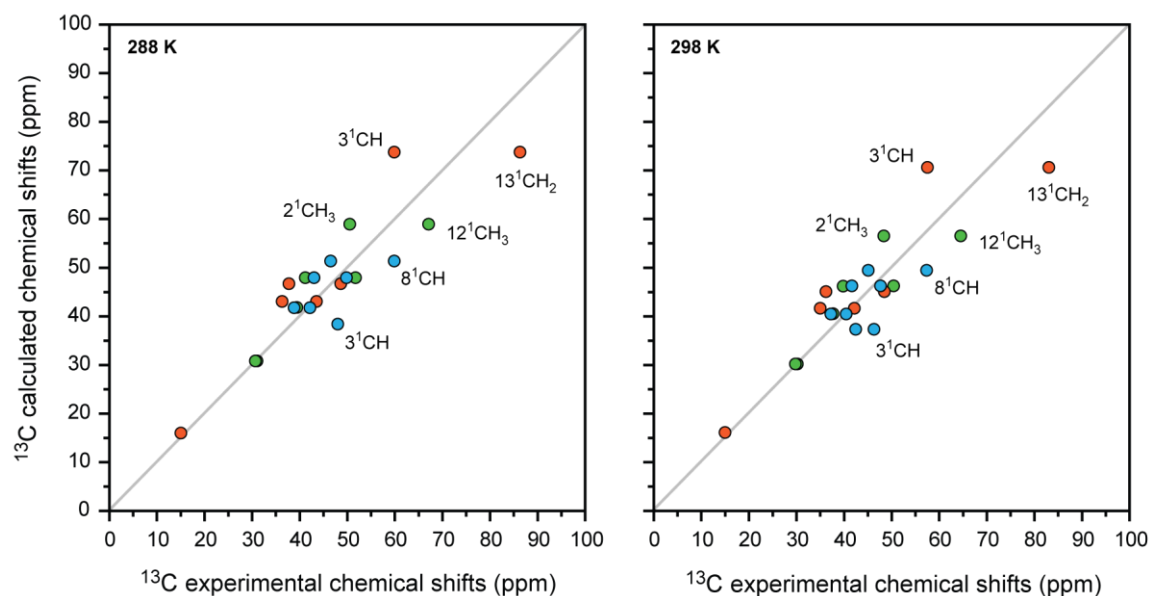


Figure 4.18 - Dispersion of the experimental and calculated ^{13}C Fermi contact shifts of the heme α -substituents of PpcF, at pH 7 (15 and 25 °C). The experimental Fermi contact shifts were obtained by subtracting the respective diamagnetic references (12.1, 36.5 and 22.5 ppm for heme methyls, thioethers and α -propionates, respectively) [17]. The data points are colored in green, orange and blue for the heme α -substituents of hemes I, III and IV, respectively.

The results obtained showed that the orientations and magnetic properties of the heme axial histidine residues are different for each heme. In the case of hemes I and IV, the axial histidine ring planes are almost perpendicular to each other, with β values close to 90° (Figure 4.17). On the other hand, in heme III, the histidine ring planes are related by a $\sim 45^\circ$ angle. These features are represented in Figure 4.17. In this figure, the length of the line representing the orientation of the rhombic perturbation (θ) is proportional to the magnitude of the energy splitting between the heme frontier molecular orbitals (ΔE). In hemes I and IV, the rhombic perturbation is oriented towards the N_C - N_D edge of the heme, whereas in heme III, it orients towards the N_B - N_C edge of the heme, all towards the center of the heme core [8]. Overall, the orientations and magnetic properties of the heme groups of PpcF are within the range of the values previously determined for the cytochromes from the PpcA-family of *G. sulfurreducens* and PpcA from *G. metallireducens* (Table 4.7). Regarding the structural properties determined in the present Thesis, the angle between the axial histidine ring planes (β), with exception of heme I of PpcD from *G. sulfurreducens*, their standard deviations are small (5° , 7° and 5° for hemes I, III and IV, respectively). The nearly parallel orientation of the heme I axial histidine rings planes in PpcD was attributed to structural variations involving residues 16–41, caused by the presence of an additional threonine residue after the heme I binding motif when compared to the other homologs [4]. A comparative analysis of the axial ligand orientations is indicated in Figure 4.19.

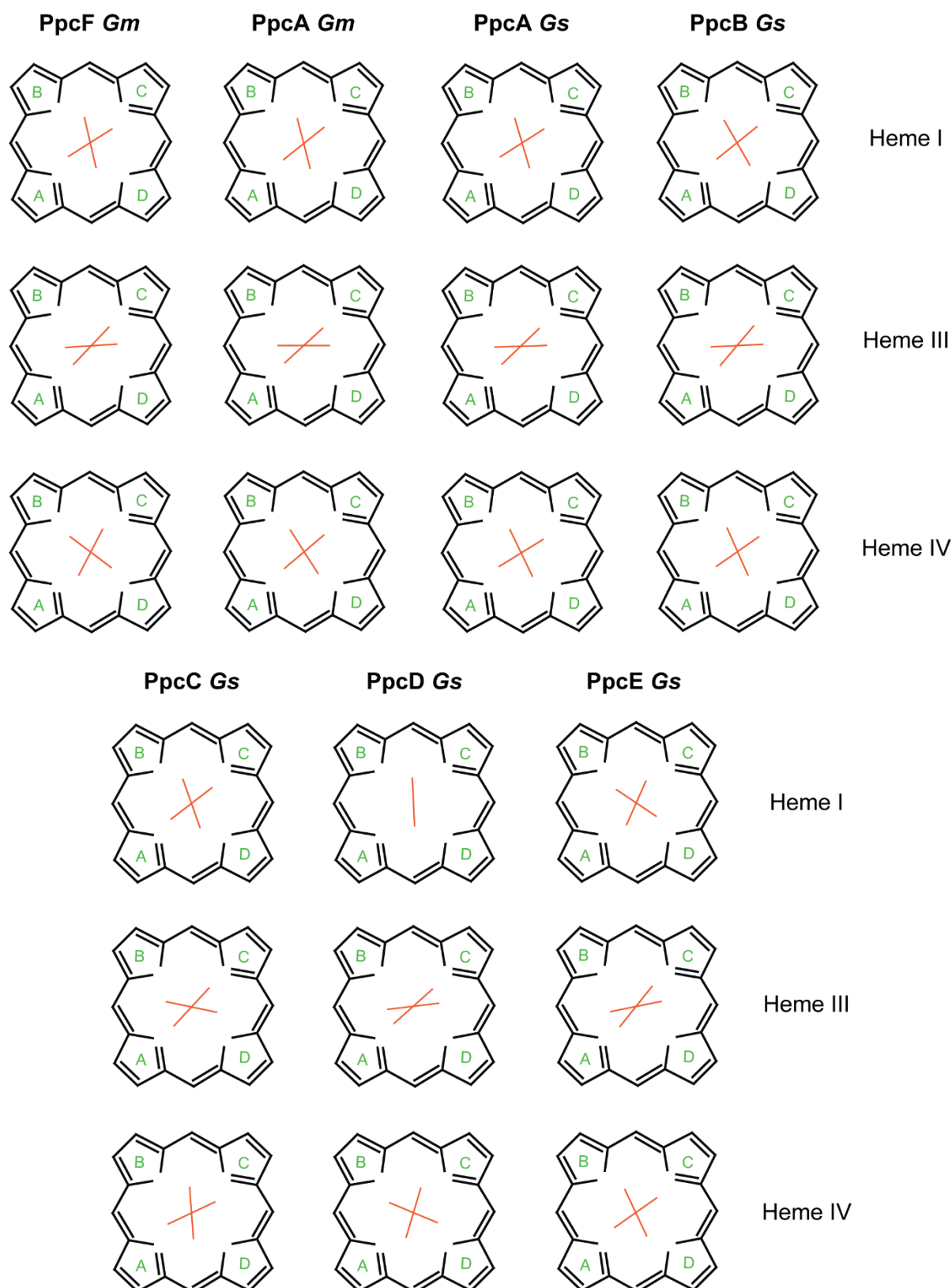


Figure 4.19 - Conservation of the orientation of the heme axial ligands of PpcA and PpcF from *G. metallireducens*, and PpcA-E from *G. sulfurreducens*. The orientations of the axial histidine planes are represented in orange. The heme pyrrole rings are identified according to the Brookhaven Protein Data Bank standard atom nomenclature for metal-substituted protohemes. The axes are defined relative to the N_C - N_A vector, with a positive angle for a rotation in the counterclockwise direction.

4.5.1.4 Conserved heme core and axial ligand geometry linked to electron transfer properties

The conservation of the heme axial ligand geometries may play a crucial role in maintaining the Fe–Fe distances and the angles between the heme porphyrin rings in these cytochromes, thus assuring a fast and efficient electron transfer between the different hemes. Moreover, the conservation of these structural motifs is of utmost importance to establish the heme-heme redox interactions that ultimately modulate the heme reduction potential values of each redox center. Heme-heme redox interactions are observable in multiredox center proteins and measure the effect of the oxidation state of one redox center in the reduction potential of its neighbors [32]. These interactions typically have positive energy values, indicating that the oxidation of a particular heme is expected to hinder the oxidation of its neighbor(s). The heme-heme redox interaction values were previously determined for several PpcA-family of triheme cytochromes (Table 4.2). The considerable range of values covered by the redox interactions, indicate that the redox behavior of the hemes is not independent, instead it is modulated by the oxidation state of the closest heme(s) group(s). In fact, while being distinct for each cytochrome due to the different natures of the heme's surrounding residues, the redox interactions follow a specific pattern that correlates with the Fe–Fe distances between the different pairs of hemes. Indeed, from the existent structural data for the PpcA-family of triheme cytochromes, the Fe–Fe distances between the closest pair of hemes (I-III and III-IV) range from 11.2 to 12.6 Å, whereas for the furthest pair of hemes (I and IV) these distances range from 18.2 to 20.8 Å [4]. The fact that for all cytochromes the redox interactions are larger for the pairs of hemes I-III and III-IV and significantly lower for the pair I-IV (Table 4.2) confirms that they are dependent on the Fe–Fe distance between each redox center [32].

The contribution of the redox interactions to the midpoint reduction potential values of the hemes can be visualized by plotting their redox curves with the actual redox interaction values or considering that the hemes groups are non-interacting. As an example, such curves are indicated for the heme groups of PpcA and PpcF from *G. metallireducens* in Figure 4.20. By comparing the two sets of curves it is clear that the redox interactions are important in establishing the heme redox profiles. In fact, the individual heme redox curves simulated without redox interactions are much steeper than those simulated considering such interactions (Figure 4.20). In the absence of redox interactions, the curves follow a pure Nernst behavior. Contrarily, in the presence of the actual redox interactions the curves do not follow a Nernstian behavior because the redox behavior of one heme is modulated by the level of oxidation of its neighboring heme(s). This is particularly notable for heme III since it is the last heme to oxidize, and it has important interactions with the nearly equidistant hemes I and IV. Due to the larger distance between hemes I and IV, the total contribution of the redox interactions to the reduction potential value is smaller (continuous and dashed lines in (Figure

4.20). Overall, the redox interactions span the individual midpoint heme reduction potential values in such a way that the cytochromes are functionally active over larger redox potential ranges. In fact, the heme reduction potential values of PpcA and PpcF, considering the existence of interactions between the different hemes, vary by 103 and 101 mV, respectively, whereas in the absence of such interactions, they only span 42 and 60 mV, respectively (Figure 4.20).

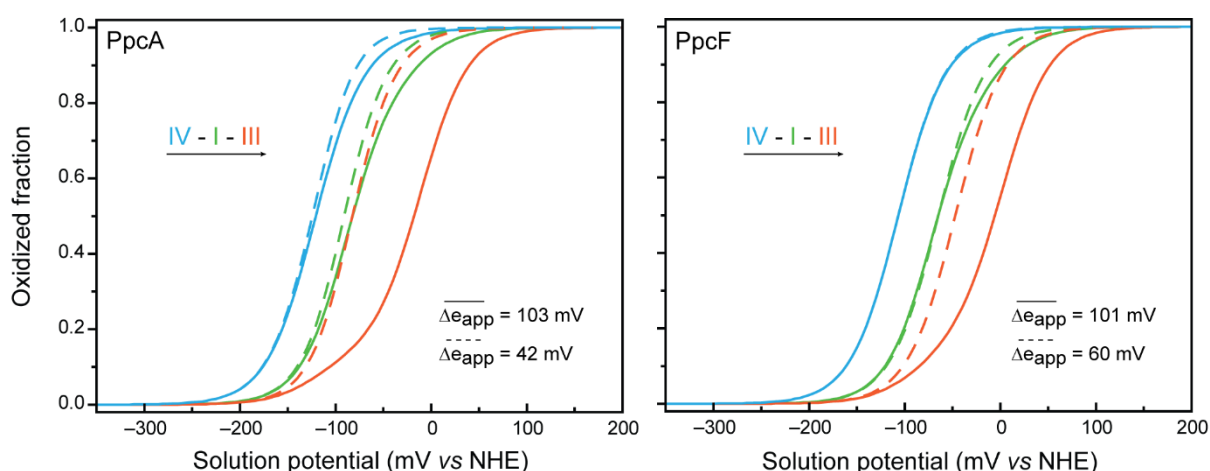


Figure 4.20 - Effect of the heme-heme redox interactions on the modulation of the individual heme redox profiles of the PpcA and PpcF cytochromes from *G. metallireducens*. The curves were calculated as a function of the solution reduction potential (relative to NHE) using the thermodynamic parameters previously obtained for PpcA [41] and PpcF [42]. The curves for hemes I (green), III (orange) and IV (blue) are represented either considering the presence of the previously determined redox interactions (solid lines) or considering non-interacting hemes (dashed lines). The differences in the midpoint reduction potential values of the first and last heme to oxidize (Δe_{app}) are indicated.

The advantage of having a unique scaffold formed by closely and strategically arranged redox centers along the polypeptide chain with high degree of sequence and structural homology is to permit their specific tuning by subtle modification of the polypeptide chains, for optimal interaction with different physiological partners and, hence, to perform an adequate function in each specific metabolic pathway. The heme reduction potential values of the PpcA-family cytochromes from *G. sulfurreducens* range from -100 to -160 mV at physiological pH [29], whereas those of PpcA and PpcF cytochromes from *G. metallireducens* function at more positive reduction potentials (0 to -120 mV) [41, 42]. The differences observed in the heme reduction potential values of these proteins, which share a high level of structural homology and sequence identity (50–80%), are a perfect example of how closely related proteins can modulate the properties of their co-factors with selective changes in their polypeptide chain, to achieve maximum effectiveness in their respective cellular pathways.

4.6 Conclusions

The bacteria *G. sulfurreducens* and *G. metallireducens* are abundant in natural environments and well-adapted to the lack of soluble electron acceptors. They naturally export electrons to extracellular electron acceptors. In this process, the periplasmic cytochromes are key electron transfer components that assure an efficient electron transfer interface between the bacteria's cytoplasm and the cell's exterior. In this Thesis, NMR and visible spectroscopic techniques were used to probe the functional properties of the periplasmic triheme cytochrome PpcF from *G. metallireducens*. In what concerns the assignment of the heme α -substituents' NMR signals of PpcF from *G. metallireducens*, it was obtained and allowed, together with a semiempirical model of the heme molecular orbitals, the orientation of the heme axial histidine planes and magnetic properties of the cytochrome to be determined.

The data obtained showed that the reduction potential values of the hemes are negative, different from each other and are modulated by a network of redox and redox-Bohr interactions. The comparison of the functional properties of cytochrome PpcF with those obtained for the homologues in *G. sulfurreducens* revealed an unprecedented functional mechanism for PpcF, which allows the heme groups to oxidize in a near independent way regardless of the pH. This is based on (i) a larger separation of the heme reduction potential values, in particular the considerable shift of the heme IV reduction potential to more negative values and (ii) a well-conserved and pH-independent stepwise oxidation of the hemes (IV-I-III) unaffected by the protonation state of the redox-Bohr center. In addition, we also showed that the reduction potential values of the heme groups in PpcF from *G. metallireducens* cover a less negative working functional redox window compared to the triheme cytochromes from *G. sulfurreducens*, a feature that might be related with the ability of *G. metallireducens* to oxidize a wider range of organic and inorganic substances. The way the protein is designed warrants an important directionality for electron transfer along the cytochrome's heme redox chain and to the electron acceptor. Given the relatively high degree of sequence and structural homology between the triheme cytochromes from *Geobacter* species [8], the data obtained constitutes an excellent example of how highly homologue proteins are specifically tuned in each microorganism. More importantly, the information obtained opens new routes for manipulating the functional properties of triheme cytochromes from *Geobacter*. In fact, based on the less conserved residues amongst the triheme cytochromes from *G. metallireducens* and *G. sulfurreducens*, rational mutants can be designed through site-directed mutagenesis. The respective impact of the mutations can be evaluated in terms of electron transfer efficiency using knock-out strains of both bacteria in order to attain an optimized strain with the favorable features for effective biotechnological application [43].

From the structural perspective, the results revealed that PpcF presents a conserved heme core architecture and axial ligand geometry compared to the cytochromes of the PpcA-family of *G. sulfurreducens* and to cytochrome PpcA from the *G. metallireducens*. The periplasmic

cytochromes from the PpcA-family are widely expressed in several different growth conditions in *Geobacter* bacteria, playing a role in the EET pathways to a multitude of final electron acceptors [6, 44]. It is, therefore, plausible to assume that the heme reduction potential values of these cytochromes reflect the overall redox behavior of these bacteria [45-47]. Based on the results obtained in the recent years, it seems that the PpcA-family cytochromes from the *G. sulfurreducens* and *G. metallireducens* bacteria diverged and developed distinct metabolic advantages. This diversity arises from genetic recombination of key residues modulating heme properties, while maintaining the overall heme core architecture. The heme arrangement and the inherent heme redox cooperativities must, therefore, be crucial for the versatile and efficient electron transfer shown by these bacteria.

4.7 References

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| 5.

**EXPRESSION OF THE PilA PROTEIN
FROM *G. metallireducens***

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5. Expression of the PilA protein from *G. metallireducens*

5.1 Introduction

The study of the interactions and electron transfer reactions between the protein PilA and the periplasmic triheme cytochromes was compromised during the time course of the Thesis by the discover that pili are in fact formed by the association of heterodimeric subunits of PilA-N and PilA-C [1] and, even more important, by the finding that PilA-N-C filaments are part of the secretion system of bacterial electroconductive filaments made by the polymeric assemble of *c*-type cytochrome subunits [1]. Therefore, it was demonstrated that pili are not directly involved in the EET. One alternative to the initial plan would be to derive the research toward the new discovered *c*-type cytochrome polymers. However, until now it was not possible to express these filaments in *E. coli* and the yields obtained from their production in *Geobacter* cells are still very low to permit such studies. Nevertheless, in this Chapter it is described the production attempts carried out for PilA protein from *G. metallireducens* during the time course of this Thesis.

5.2 Methods

The attempts to obtain the PilA protein from *G. metallireducens* were based on the work of Reardon and Mueller [2].

5.2.1 Cloning of the gene of the PilA protein

PilA protein from *G. metallireducens* was cloned in pHTP14 (NZYTech) expressing vector (Figure 5.1A), containing ketosteroid isomerase (KSI) and a His-tag (Figure 5.1B). The plasmid pHTP14 was designed for the cloning and expression of high levels of recombinant proteins in *E. coli*. KSI is a hydrophobic protein, with a strong tendency to accumulate in inclusion bodies. Therefore, recombinant proteins are commonly expressed in fusion with KSI to promote solubility and folding of its fusion partners. The KSI protein and target peptide sequences are expressed with intervening methionine residues, so that cyanogen bromide (CnBr) cleavage separates out KSI and the individual peptides.

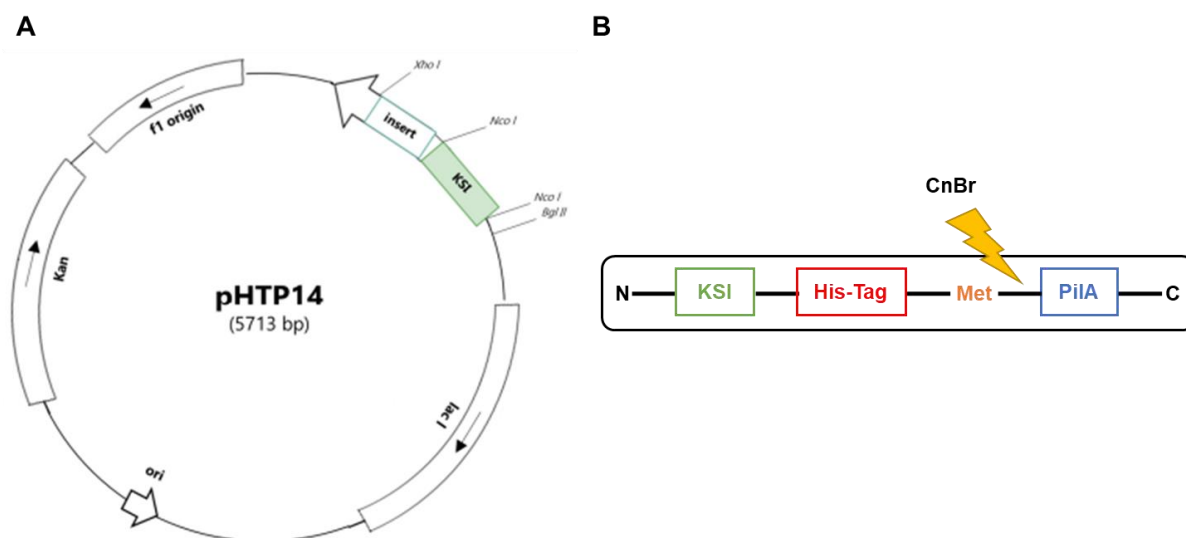


Figure 5.1 - pHTP14 vector. (A) Vector map (NZYTech). (B) Desired construct.

The primers were designed with a region that hybridizes in the pHTP14 vector (overhangs) and another region that hybridizes in the genomic DNA, from where the gene of interest will be obtained. The sequence of the gene of interest, *gmet_1399*, was picked from the genomic DNA in agreement with <http://www.genome.jp/kegg/genome.html>. Also, a methionine residue was added before the protein sequence to later permit the removal of the His-tag from the PilA protein with CnBr cleavage (Figure 5.1B). Primer design followed the instructions on NZYEasy cloning kit (NZYTech) protocol. Primers were checked for dimer formation using the Thermo Scientific tool for primer analysis (<https://www.thermofisher.com>). The primers used in the procedure were produced by Invitrogen and are presented in Table 5.1.

Table 5.1 - Sequence of the DNA primers used to clone PilA protein from *G. metallireducens* in the pHTP14 plasmid. The melting temperatures (T_m) for each primer and the annealing temperatures were calculated from the Thermo Scientific web tool (<https://www.thermofisher.com>). The sequences complementary to plasmid are represented in blue and the insert sequence are represented in green and were designed using the restricting free cloning website (<https://www.rf-cloning.org>).

	DNA sequence	T_m (°C)	Annealing Temperature (°C)
Forward primer	5' TCAGCAAGGGCTGAGGATG TTCACCTCATCGAGC 3'	54.5	58.4
Reverse primer	5' TCAGCGGAAGCTGAGGTTAATT CGGATAAAATTGGTGTTC 3'	57.0	

The PCR samples were prepared as presented in Table 5.2 and the PCR cycling conditions are indicated in Table 5.3. Two primer concentrations were tested (0.5 and 0.25 μ M). Negative controls, without template DNA, were also performed.

Table 5.2 - Composition of the final PCR mix. The Phusion DNA polymerase, Phusion buffer and nucleotides are from Thermo Scientific. 1 U is the amount of enzyme needed to convert in one minute 1 μ mole of substrate.

PCR reaction mix components	Final concentration
Phusion High Fidelity Buffer	1x
Nucleotides	200 μ M
Forward Primer	0.5 / 0.25 μ M
Reverse Primer	0.5 / 0.25 μ M
Template DNA	0.5 ng/ μ L
Phusion DNA Polymerase	0.02 U/ μ L

Table 5.3 - Cycling conditions of the first PCR. The annealing/extension time was calculated according to the manufacturer's indication of 15-30 seconds/kb.

PCR step	Temperature (°C)	Time (seconds)	Cycles
Initial denaturation	98	30	1
Denaturation	98	8	34
Annealing	59	20	
Extension	72	10	
Final extension	72	600	1
Final hold	16	-	-

The size of the PCR products was confirmed by 1.5% agarose gel stained with GreenSafe (NZYTech) and using GeneRuler 100 bp DNA ladder (New England BioLabs). Then, PCR products were purified using the NZYGelpure (NZYTech) and quantified with NanoDrop™ (Thermo Fisher Scientific).

After this step, NZYEasy cloning and expression kit XIV (NZYTech) was used to clone the PCR product into the pHTP14 vector. In this kit cloning proceeds in a single ligase-independent reaction mediated by the NZYEasy enzyme mix. Vector-complementary overhangs containing a specific sequence recognized by the NZYEasy enzyme are incorporated in the PCR product by using primers with appropriate 5' extensions. The circular recombinant vector containing the fragment of interest is obtained by transforming the annealed plasmid DNA into competent *E. coli* cells. This kit allows achieving high cloning efficiency (80 - 100%) and does not require the use of DNA ligases. In addition, no further treatment (*e.g.* restriction digestion, phosphorylation, or blunt-end polishing) of the inserts is required. In this step, the composition of the PCR mixtures used and PCR cycling conditions were followed according to NZYEasy cloning and expression kit XIV protocol.

E. coli DH5α competent cells were transformed with the constructed vectors and plated for selection in Luria-Bertani (LB) medium supplemented with kanamycin 50 µg/ml (NZYTech). Proper controls were performed. The resulting colonies were screened by colony PCR [3] (with T7 primers from VWR and Taq polymerase (NZYTaq II DNA polymerase) from NZYTech) with the PCR mixture composition and PCR cycling conditions represented in Table 5.4 and Table 5.5, respectively, and analyzed by 1.5% agarose gel electrophoresis.

Table 5.4 - Composition of the final PCR mix. The Taq polymerase and Taq reaction buffer are from NZYTech. Nucleotides are from Thermo Scientific. 1 U is the amount of enzyme needed to convert in one minute 1 µmole of substrate.

PCR reaction mix components	Volume (20 µL)
Taq reaction buffer	2 µL
Nucleotides	0.4 of 10 U/µL
Forward Primer T7	0.2 µL of 50 µM
Reverse Primer T7	0.2 µL of 50 µM
Taq polymerase	0.25 µL of 5 U/µL
MiliQ water	16.95 µL

Table 5.5 - Cycling conditions of colony PCR.

PCR step	Temperature (°C)	Time (seconds)	Cycles
Initial denaturation	95	120	1
Denaturation	95	30	29
Annealing	50	30	
Extension	72	60	
Final extension	72	600	1
Final hold	16	-	-

The colonies with PCR products of the correct size were grown in liquid LB medium supplemented with kanamycin for plasmid extraction and purification with NZYMiniprep kit. However, the plasmid yield was not sufficient and GeneJET Plasmid Midiprep Kit (Thermo Scientific) was then used. Finally, plasmid DNA sequence was confirmed by DNA sequencing performed by STAB Vida.

5.2.2 Expression of the PilA protein

E. coli C41 (DE3) cells were transformed with the pHTP14 plasmid harbouring the PilA gene from *G. metallireducens* (pHTP14_pilA) and placed on ice for 30 min. Then, the solution was kept on 42 °C for 1 min, followed by another minute on ice. 500 µL of LB medium was added and cells were incubated for 1h at 37 °C and 200 rpm. Afterwards, cells were inoculated in LB solid medium, containing 15 g/L agar and supplemented with 50 µg/ml kanamycin, overnight at 37 °C. One colony was added to 5 mL of LB liquid medium supplemented with the same amount of kanamycin and incubated at 37 °C and 200 rpm. Approximately 7 hours later, 500 µL of this culture was transferred to 50 mL of LB medium (supplemented with the same antibiotic) and grown overnight at 37 °C and 200 rpm. 10 mL of the culture was transferred to 1 L of LB medium with kanamycin (50 µg/ml) and incubated at 37 °C and 180 rpm until it reaches OD_{600nm} ~ 0.6. At this stage, expression of PilA protein was induced with 1 mM IPTG and cells were incubated at 37 °C and 180 rpm for 4 to 6 hours. Cells were harvested by centrifugation at 9000 *xg* and resuspended in NZY bacterial cell lysis buffer (NZYTech) and stored at -20 °C. Thawed cells were incubated with lysozyme and DNase, as recommend in NZY bacterial cell lysis buffer protocol, for 1 h. Then, the insoluble inclusion bodies were separated from the soluble cellular components by centrifugation at 15000 *xg* for 15 min at 4°C and solubilized in loading buffer containing 6 M guanidine-HCL, 10 mM imidazole and 50 mM sodium phosphate at pH 8.

5.2.3 Purification of the PilA protein

The fusion protein (PilA protein from *G. metallireducens* plus His-tag and KSI) was separated by affinity chromatography since it contains a His-tag, using a Econo-Pac gravity flow column (Bio-Rad) of 20 mL packed with HisPur Ni-NTA resin (Thermo Scientific). The column was previously equilibrated with 6 M guanidine-HCL, 10 mM imidazole and 50 mM sodium phosphate at pH 8. The elution was performed with a step gradient from 10 mM to 250 mM imidazole and 1% β -mercaptoethanol was added to collected samples, which were then stored overnight at 4 °C.

The fractions containing the fusion protein were dialyzed against MiliQ water to precipitate and were pelleted by ultracentrifugation at 14700 $\times g$ for 1 h at 4°C. The pellet was resuspended in buffer containing 6 M guanidine-HCL, 50 mM Tris-HCL, 500 mM NaCl and 0.5 M HCL.

To perform the cleavage of the fusion protein from the PilA protein, the resuspended pellet reacted with 1 M cyanogen bromide for 2 h. The cleaved product was dialyzed against MiliQ water and lyophilized. Then, the cleaved PilA was resuspended in 6 M guanidine-HCL, 25 mM imidazole and 50 mM sodium phosphate at pH 8 buffer and separated from uncleaved fusion protein using a Econo-Pac gravity flow column of 20 mL packed with HisPur Ni-NTA resin (Thermo Scientific) and equilibrated with 6 M guanidine-HCL, 25 mM imidazole and 50 mM sodium phosphate at pH 8 buffer.

15% acrylamide/bis-acrylamide or 15% tris-tricine SDS-PAGE stained with BlueSafe (NZYTech) using the molecular weight markers NZYColour Protein Marker II (NZYTech) or Protein Plus Protein™ Dual Xtra Standards (Bio-Rad) were used between steps to evaluate the samples.

5.2.4 Refolding of the PilA protein

The purified PilA protein was dialyzed against MilliQ water and then ultracentrifuged at 14700 $\times g$ for 1 h at 4 °C. The obtained pellet was resuspended in buffer with 6 M guanidine-HCL and 50 mM sodium phosphate (pH 8) and dropwise in 1% β -octylglucopyranoside (β -OG), 50 mM sodium phosphate pH 6. Insoluble protein was removed by centrifugation at 9000 $\times g$, 15 min at 4 °C and then extensively dialyzed against MilliQ to remove β -OG. After this step, the PilA protein was lyophilized and dissolved in 200 mM dihexanoylphosphatidylcholine (DHPC).

In further studies with PilA protein expressed with plasmid pHTP14_pilA with Tobacco Etch Virus (TEV) protease cleavage sequence, protein refolding was attempted with 2% DDM (n-dodecyl- β -D-maltoside), 1% β -OG and 70 mM DHPC and was purity evaluated with 15% acrylamide/bis-acrylamide SDS-PAGE.

5.2.5 Testing the expression of the PilA protein in different *E. coli* strains

The expression of PilA protein was tested in *E. coli* strains C41 (DE3), BL21 (DE3) pLysS, Rosetta 2 (DE3) pLysS and BL21 star (DE3). Each strain was transformed with plasmid pHTP14 containing the *pilA* gene from *G. metallireducens* and grown overnight at 37 °C in 5 mL of LB medium supplemented with the respective antibiotics of each strain. 500 µL of each culture was transferred to 50 mL of LB medium (plus antibiotics) until they reach OD_{600nm}~0.6. At this point induction was achieved with 1 mM IPTG and grown for 4h at 37 °C and 160 rpm. Cultures were centrifuged at 5000 *xg* for 10 min and aliquots of each culture were collected and evaluated by 15% acrylamide/bis-acrylamide SDS-PAGE stained with BlueSafe with the protein marker Precision Plus Protein™ Dual Xtra Standards ranging between 2 and 250 kDa.

5.2.6 TEV protease cleavage site insertion

The TEV protease is a highly specific cysteine protease of 27 kDa that recognizes the sequence ENLYFQ|S with high catalytic efficiency making it an excellent option to remove affinity purification tags, such as His-tag [4].

For the insertion, the protocol followed the strategy based on Q5® Site-Directed Mutagenesis (New England BioLabs), which is indicated in Figure 5.2.



Figure 5.2 - Overview of Q5® Site-Directed Mutagenesis kit protocol. Adapted from the kit's instructions manual.

The design of the primers to include the TEV cleavage site in the pHTP14 plasmid harboring the PilA protein gene (pHTP14_pilA), Figure 5.3, was performed with resource to the New England BioLabs tool (<https://nebbasechanger.neb.com/>). Designed primers are represented in Table 5.6 and were synthesized by Invitrogen.



Figure 5.3 – Schematic representation of the desired construct. The sequence recognized by TEV protease is represented in orange.

Table 5.6 - Sequence of the DNA primers used to insert a TEV cleavage site in the pHTP14 plasmid harboring the PilA protein from *G. metallireducens* gene. The melting temperatures (T_m) for each primer and the annealing temperatures are presented. The sequences complementary to plasmid are represented in green and the insert sequence are represented in blue and were designed using the New England BioLabs tool (<https://nebasechanger.neb.com/>).

	DNA sequence	T_m (°C)	Annealing Temperature (°C)
Forward primer	5' TTTTCAGTCTTTCACC CTCATCGAGCTG 3'	64.0	65.0
Reverse primer	5' TACAGGTTTCCATCCT CAGCCCTTGCTG 3'	66.0	

The pHTP14_pilA template was amplified by PCR using the designed primers (Table 5.6). The PCR mix reactions used are described in Table 5.7 and two different amounts of template DNA were tested. The PCR program is described in Table 5.8. The size of the PCR products was confirmed by 0.8% agarose gel using GeneRuler 1 kb DNA ladder (New England BioLabs) and to the PCR products with the right size were added a mixture of kinase, ligase and DpnI from Q5® Site-Directed Mutagenesis kit for circularization and template removal. Then, colony PCR was performed using the same methodology describe in section 5.2.1. 1% agarose gel electrophoresis was used to screen the colonies and select the ones with the right size which its DNA was after extracted with GeneJET Plasmid Midiprep Kit according to the manufacturer's instructions and the insertion of the TEV cleavage site was confirmed by DNA sequencing performed by STAB VIDA.

Table 5.7 - Composition of the PCR mix for site-directed mutagenesis. The Phusion DNA polymerase, Phusion buffer and nucleotides are from Thermo Scientific. 1 U is the amount of enzyme needed to convert in one minute 1 μ mole of substrate.

PCR reaction mix components	Final concentration
Phusion High Fidelity Buffer	1x
Nucleotides	200 μ M
Forward Primer	0.5 μ M
Reverse Primer	0.5 μ M
Template DNA	0.2 / 0.5 ng/ μ L
Phusion DNA Polymerase	0.02 U/ μ L

Table 5.8 - Cycling conditions for site-directed mutagenesis. The annealing/extension time was calculated according to the manufacturer's indication of 15-30 seconds/kb.

PCR step	Temperature (°C)	Time (seconds)	Cycles
Initial denaturation	98	30	1
Denaturation	98	10	30
Annealing	65	20	
Extension	72	120	
Final extension	72	600	1
Final hold	16	-	-

5.2.7 Production of the TEV protease

Rosetta 2 (DE3) pLysS *E. coli* cells were transformed with the pRK793 plasmid that contains the TEV protease S219V mutant with an N-terminal His-tag. The expression was performed as described by Tropea, Cherry and Waugh [5]. Cells were grown in LB medium supplemented with 34 µg/mL CLO and 100 µg/mL AMP at 37 °C, 200 rpm, up to OD_{600nm}~1.5. At this stage, protein production was induced with 1 mM IPTG, and temperature was reduced to 30 °C. After 4h, cells were harvested by centrifugation at 9000 *xg*, 20 min, 4 °C, and resuspended in 10 mL of 50 mM sodium phosphate buffer pH 8, 100 mM NaCl, 10% glycerol and 25 mM imidazole, per gram of cells, and frozen.

Thawed cells were ultrasonicated with 3 cycles of 2 min on plus 1 min off, on an Ultrasonic homogenizer (Branson) regulated for 65% of amplitude and in the presence of DNase I, 2 mM benzamidine and 1 mM phenylmethanesulfonylfluoride (PMSF).

The first purification step consisted of an affinity chromatography on a 5 mL Ni-NTA HisTrap HP column (GE Healthcare) equilibrated with the lysis buffer. An imidazole gradient of 50 mL from 25 to 500 mM imidazole was used to elute the protein. 12.5% acrylamide/bis-acrylamide SDS-PAGE was performed to analyze collected fractions. For the second purification step (molecular exclusion chromatography) 1 mM DTT and 1 mM EDTA was added to the sample, and it was concentrated to 3 mL in Amicon Ultra centrifugal filter units (Ultra-15, MWCO 10 kDa, Millipore) and injected in a XK 16/70 Superdex 75 (GE Healthcare) molecular exclusion column, equilibrated with 25 mM sodium phosphate buffer pH 8, 200 mM NaCl, 10% glycerol, 2 mM EDTA and 10 mM DTT. 12.5% acrylamide/bis-acrylamide SDS-PAGE was performed to pool the appropriate fractions and pure protein was concentrated to 1 mg/mL, flash freeze with liquid nitrogen and stored at -80 °C in 1 mL aliquots.

5.3 Results and discussion

5.3.1 Cloning, expression and purification of PilA protein

As described in section 5.2.1, the size of the PCR products was confirmed by 1.5% agarose gel. In Figure 5.4, Lanes 2 and 3 correspond to the PCR product with 0.5 and 0.25 μM of primers which both present the correct size of the PCR product (183 bp) and for that reason both PCR mixtures were used forward.

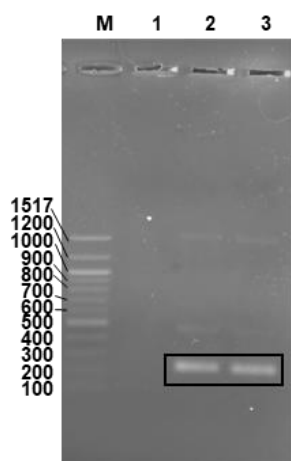


Figure 5.4 – Agarose gel electrophoresis of PCR products in 1.5% agarose gel, 1x Tris-acetate-EDTA (TAE) buffer. Lane M corresponds to the 100 bp DNA ladder. Lane 1 corresponds to the negative control. Lane 2 and 3 corresponds to the PCR product with 0.5 and 0.25 μM of primers concentration, respectively. The DNA band corresponding to the PCR product (expected size of 183 bp) is highlighted by a black box.

In what concerns PCR colony, the size of the PCR product of the selected colony was also confirmed by 1.5% agarose gel electrophoresis (Figure 5.5) and since it possesses the correct size – 888 bp, we moved on to plasmid extraction. However, the common method, miniprep was not sufficient to extract the desired amount of plasmid so we resorted to midiprep instead where a higher amount of plasmid is obtained. The DNA sequence of the obtained plasmid was confirmed by DNA sequencing and then we moved for the protein overexpression

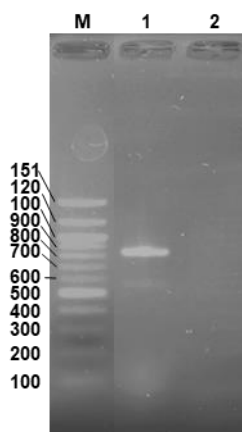


Figure 5.5 - Agarose gel electrophoresis of PCR products in 1.5% agarose gel, 1x TAE buffer. Lane M corresponds to the 100 bp DNA ladder. Lane 1 corresponds to the PCR product (expected size of 888 bp). Lane 2 corresponds to the negative control.

At first, the expression of the PilA protein was performed in *E. coli* C41 (DE3), since these are the cells used by Reardon and Mueller [2]. After transformation and plating, cell growth was taken until an $OD_{600nm} \sim 0.6$ was reached and induction was started. 4h after induction, cells were collected and lysed. Then, an affinity chromatography was performed and SDS-PAGE stained with BlueSafe allowed to infer about the obtained samples (Figure 5.6).

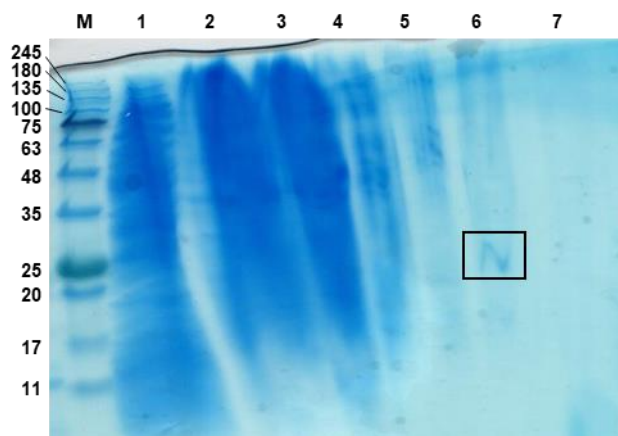


Figure 5.6 – 15% acrylamide/bis-acrylamide SDS-PAGE analysis of the affinity chromatography performed in Ni-NTA beads to purify the fusion protein from growth in *E. coli* C41 (DE3) cells. Lane M corresponds to the molecular weight marker NZYColour Protein Marker II. Lane 1 corresponds to the aliquot collected before lysis with NZY bacterial cell lysis buffer. Lane 2 corresponds to the load injected on the column. Lane 3 corresponds to the flow-through. Lanes 4 and 5 corresponds to the fractions eluted with 10 and 25 mM imidazole, respectively. Lanes 6 and 7 corresponds to the first and second elution with 250 mM imidazole, respectively. The band of interest is highlighted by a black box.

By analyzing the results, a band with the expected weight (22.4 kDa), highlighted in Figure 5.6, of the fusion protein was present in the first elution with 250 mM imidazole. However, this was the only time the protein could be monitored in an SDS-PAGE. Thus,

obtained samples along the overexpression attempt were analyzed by measuring the optical density at 280 nm. However, none of them the presence of protein was detected.

Hence, we proceeded to expression tests in different strains of *E. coli* cells, namely BL21 (DE3) pLysS, Rosetta 2 (DE3) pLysS and BL21 star (DE3) versus C41 (DE3). The SDS-PAGE is not presented but better results were observed for *E. coli* strain Rosetta 2 (DE3) pLysS. Thus, the same protocol that was performed with *E. coli* C41 (DE3) cells was now reproduced with *E. coli* Rosetta 2 (DE3) cells pLysS. The 15% SDS-PAGE performed for obtained fractions of the affinity chromatography performed with Ni-NTA beads to purify the fusion protein is presented in Figure 5.7.

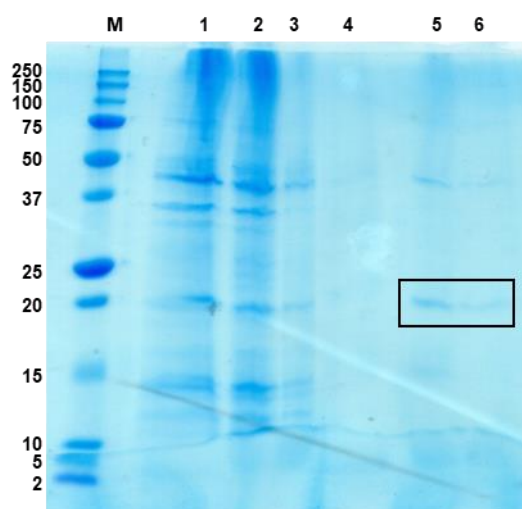


Figure 5.7 – 15% acrylamide/bis-acrylamide SDS-PAGE analysis of the affinity chromatography performed in Ni-NTA beads to purify the fusion protein from growth in *E. coli* Rosetta 2 (DE3) pLysS cells. Lane M corresponds to the molecular weight marker Protein Plus Protein™ Dual Xtra Standards. Lane 1 corresponds to the load injected on the column. Lane 2 corresponds to the flow-through. Lanes 3 and 4 corresponds to the fractions eluted with 10 and 25 mM imidazole, respectively. Lanes 5 and 6 corresponds to the first and second elution with 250 mM imidazole, respectively. The bands corresponding to the fusion protein are highlighted by a black box.

The band corresponding to the fusion protein (22.4 kDa) is observable in the fraction eluted with 250 mM imidazole as expected. These fractions were combined, dialyzed against milliQ water and ultracentrifuged to pellet the inclusion bodies which were then resuspended and cleaved with cyanogen bromide. Every time the cleavage reaction with cyanogen bromide was attempted, the protein could never be monitored after that step. Therefore, although Reardon and Mueller suggest a 2h reaction, we also tested reaction times of 4, 6 and 8 hours. 15% tricine SDS-PAGE were used to evaluate the cleavage process (Figure 5.8) since usually tricine gels show better results than acrylamide/bis-acrylamide gels to analyze samples with low weight.

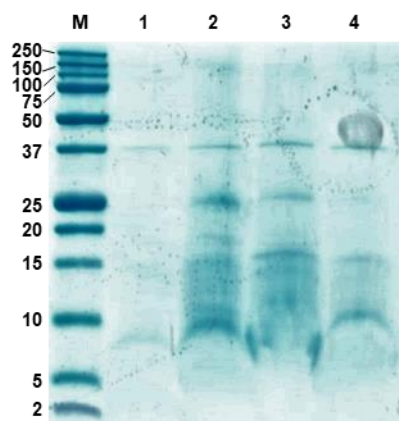


Figure 5.8 – 15% tris-tricine SDS-PAGE analysis of cleavage with cyanogen bromide. Lane M corresponds to the molecular weight marker Protein Plus Protein™ Dual Xtra Standards. Lanes 1, 2, 3 and 4 corresponds to samples after 2, 4, 8 and 6h of cleavage reaction with cyanogen bromide.

While the fusion protein (PilA protein from *G. metallireducens* plus His-tag and KSI) has 22.4 kDa, PilA protein has only 6.6 kDa, so it would be expected that after the cleavage reaction and purification of the PilA protein with affinity chromatography only one band with 6.6 kDa would be observed in the collected fraction (wash). However, we are faced with samples that have several bands of different molecular weights, which suggests that many unwanted fragments are being originated.

From this point, we decided to abort the cyanogen bromide cleavage reaction and proceeded to insert a TEV cleavage site into the plasmid sequence and consequently produce the TEV protease and then to try to perform the cleavage of the fusion protein with TEV. The first step was the insertion of the TEV protease recognition sequence in the pHTP14_pilA plasmid right before the *pilA* gene in order to be able to separate PilA protein from KSI which is adjacent to the His-tag. The size of the PCR products was confirmed by 0.8% agarose gel (Figure 5.9) and it confirmed that both PCR products (0.2 / 0.5 ng/μL) were the correct size, since pHTP14_pilA has 5896 bp and TEV recognition site has 21 bp, so the expected band was 5917 bp.

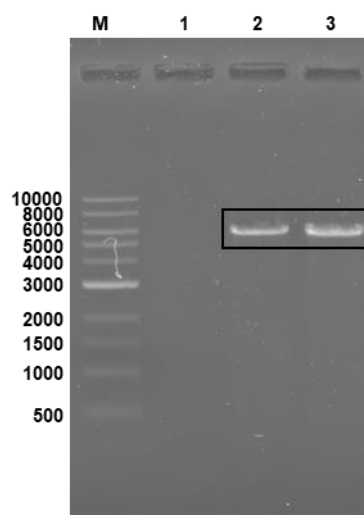


Figure 5.9 - Agarose gel electrophoresis of PCR products in 0.8% agarose gel, 1x TAE buffer. Lane M corresponds to the 1 kb DNA ladder. Lane 1 corresponds to the negative control. Lane 2 and 3 corresponds to the PCR product with 0.2 and 0.5 μ M of primers concentration, respectively. The DNA band corresponding to the PCR product (expected size of 5917 bp) is highlighted by a black box.

After colony PCR, agarose gel electrophoresis, this time with 1% agarose (Figure 5.10), was also performed to screen the colonies and select the ones with the right size (lanes 1, 3, 4, 5, 9, 10, 11 and 13) whose DNA sequence (5 and 13) was then confirmed by DNA sequencing. Hence, this plasmid was transformed in *E. coli* Rosetta 2 (DE3) pLysS cells and the same protocol for the expression of the PilA protein was followed up to the His-tag cleavage step.

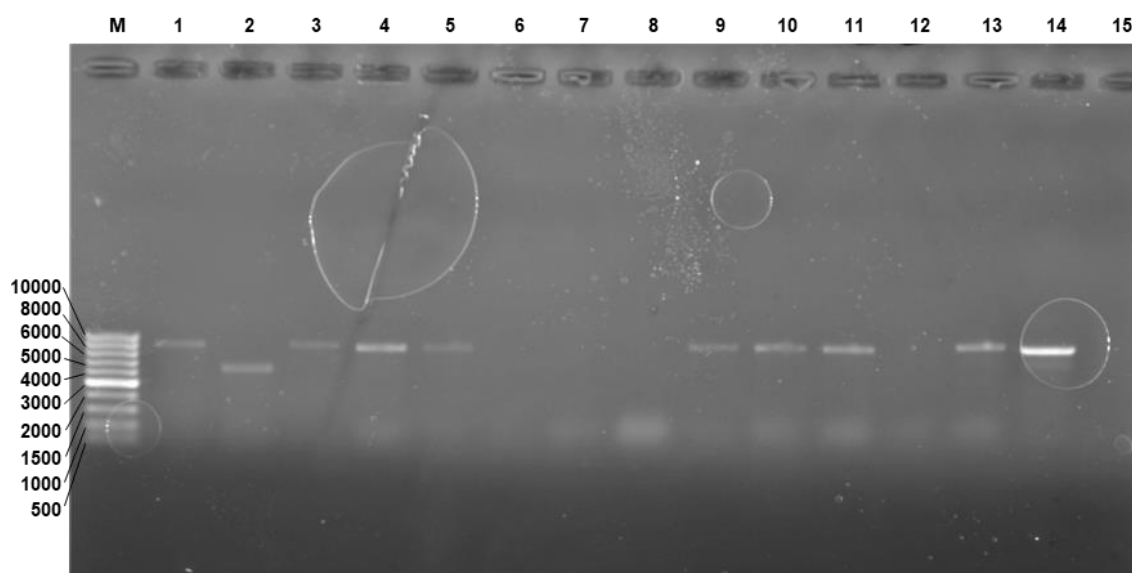


Figure 5.10 - Agarose gel electrophoresis of PCR products in 1.5% agarose gel, 1x TAE buffer. Lane M corresponds to the 1 kb DNA ladder. Lanes 1 to 13 corresponds to the screened colonies. Lane 14 corresponds to the positive control. Lane 15 corresponds to the negative control.

In what concerns the TEV protease production, the first chromatographic step consisted of an affinity chromatography since it had a His-tag attached. However, the obtained chromatogram (Figure 5.11) was not straightforward to decide which fractions to put together since a broad band and not a narrow peak was observed in the elution profile. SDS-PAGE (Figure 5.12) was used to analyze the collected samples and decide which ones to pull together for the next chromatographic step and since all fractions demonstrated to have TEV protease (27 kDa from TEV plus approximately 2 kDa from His-tag), all of them were put together to be injected in molecular exclusion chromatography column.

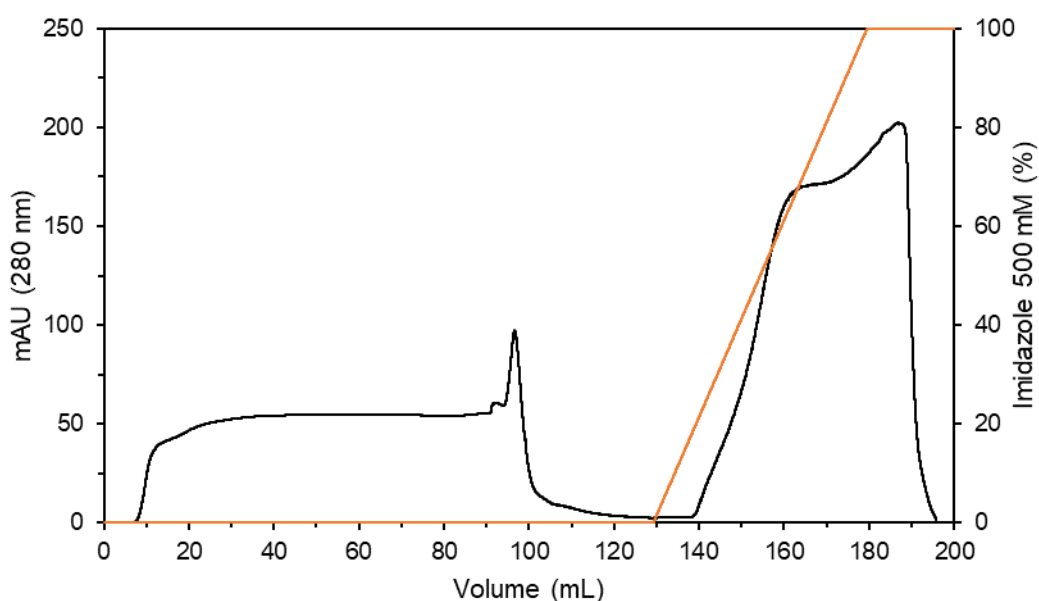


Figure 5.11 - Affinity chromatography elution profile of TEV protease on 5 mL Ni-NTA HisTrap HP column. The primary and secondary y-axis report the variation of absorbance at 280 nm (black line) and the imidazole gradient profile (orange line), respectively.

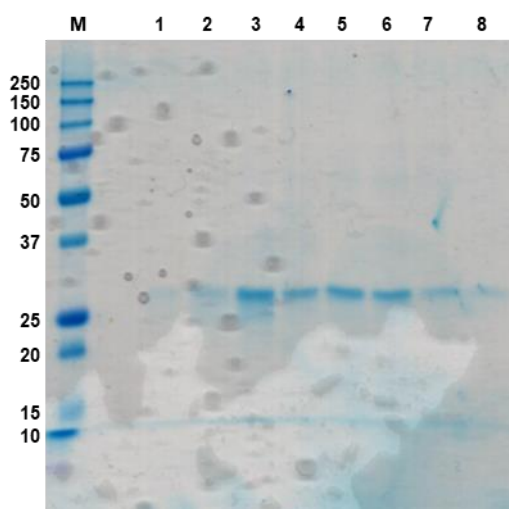


Figure 5.12 – 12.5% acrylamide/bis-acrylamide SDS-PAGE analysis of collected fractions from TEV affinity chromatography. Lane M corresponds to the molecular weight marker Protein Plus Protein™ Dual Xtra Standards. Lanes 1 to 8 corresponds to collected fractions from TEV affinity chromatography.

The fractions corresponding to TEV protease were loaded onto a XK 16/70 Superdex 75 molecular exclusion column and the analysis of the fractions obtained by SDS-PAGE concluded that the protein was pure (Figure 5.13). Using this procedure, the yield obtained was approximately 1 mg/L of cell culture.

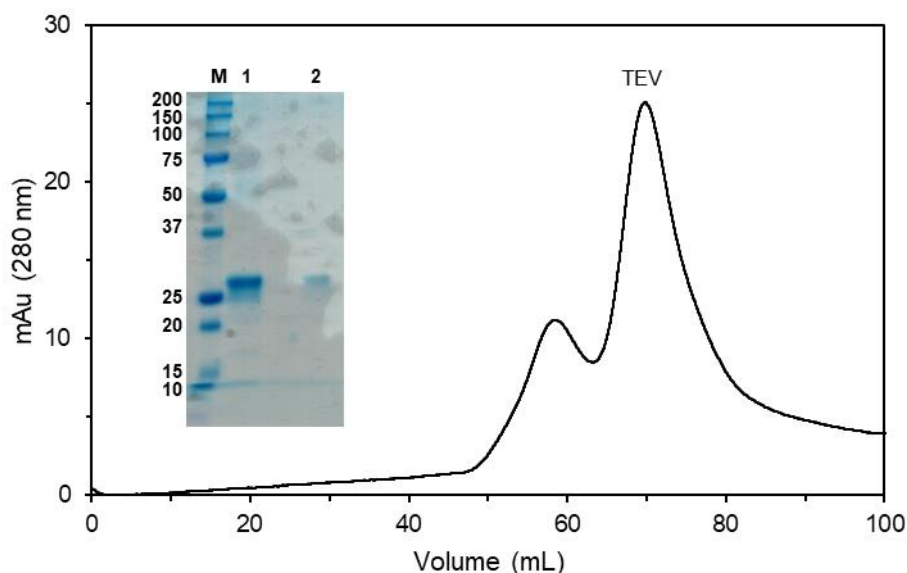


Figure 5.13 - Molecular exchange chromatography elution profile of TEV and SDS-PAGE of collected fractions. The peak corresponding to the TEV fractions is identified. Lane M corresponds to the molecular weight marker (Protein Plus Protein™ Dual Xtra Standards, from Bio-Rad). Lane 1 corresponds to the load sample and lane 2 corresponds to the collected fractions of pure TEV.

Produced PilA protein with TEV cleavage site, after affinity chromatography, was dialyzed, centrifuged and resuspended in 50 mM sodium phosphate pH 8, 6 M guanidine-HCL and refolded by dropwise in 50 mM sodium phosphate pH 8 with 2% *t*-octylphenoxypolyethoxyethanol (Triton X-100). TEV protease, 1 mM DTT and 0.5 mM EDTA was added to cleave the fusion protein overnight at 4 °C. However, analysis of the SDS-PAGE of samples before and after cleavage with TEV (Figure 5.14) demonstrate that the cleavage reaction was not efficient since in the samples before and after the cleavage reaction the only difference is the presence of TEV in the last.

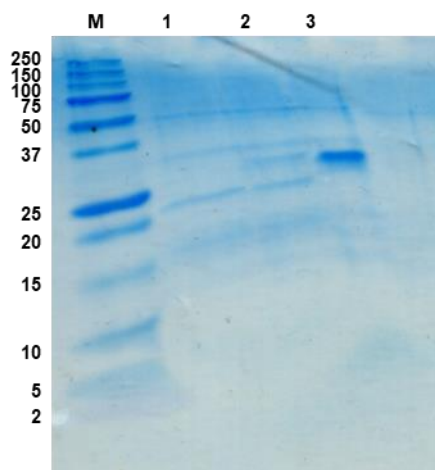


Figure 5.14 - 15% acrylamide/bis-acrylamide SDS-PAGE analysis of cleavage reaction with TEV protease in Triton X-100. Lane M corresponds to the molecular weight marker Protein Plus Protein™ Dual Xtra Standards. Lane 1 corresponds to fusion protein before cleavage. Lane 2 corresponds to fusion protein after cleavage. Lane 3 corresponds to TEV protease.

Considering that cleavage reaction with TEV protease was not efficient, instead of trying to refold the fusion protein in Triton X-100, we tried refold in 2% DDM, 1% β -OG and 70 mM DHPC, since the TEV activity is not affected with these detergents [6]. The SDS-PAGE of the samples is depicted in Figure 5.15. and demonstrate that cleavage reaction was inefficient since samples remain unaltered after reaction with TEV.

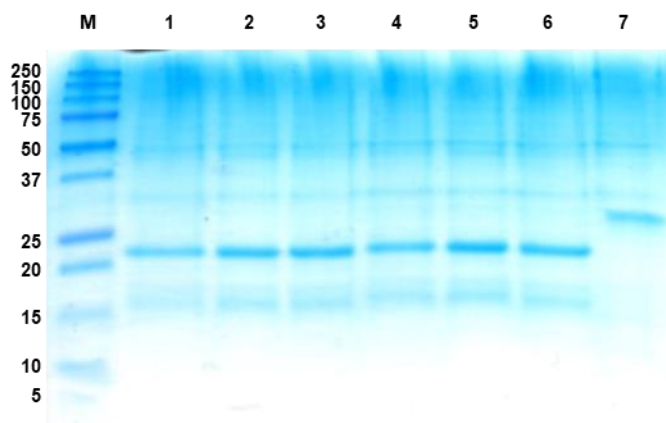


Figure 5.15 - 15% acrylamide/bis-acrylamide SDS-PAGE analysis of cleavage reaction with TEV protease in different detergents. Lane M corresponds to the molecular weight marker Protein Plus Protein™ Dual Xtra Standards. Lanes 1, 2 and 3 corresponds to sample before cleavage reaction with TEV protease in the presence of DHPC, DDM and β -OG, respectively. Lanes 4, 5 and 6 corresponds to sample after cleavage reaction with TEV protease in the presence of DHPC, DDM and β -OG, respectively. Lane 7 corresponds to TEV protease.

5.4 Conclusions

The gene coding for the PilA protein from *G. metallireducens* was successfully cloned in the pHTP14 plasmid that harbors KSI and a His-tag. Expression was tested in *E. coli* strains C41 (DE3), BL21 (DE3) pLysS, Rosetta 2 (DE3) pLysS and BL21 star (DE3) cells and the more promising results were obtained with *E. coli* Rosetta 2 (DE3) pLysS. The PilA protein was produced as a fusion protein and to cleave it from the KSI protein, cleavage with CnBr was attempted, but was not successful. To overcome this difficulty a TEV protease cleavage site was introduced between the His-tag and the PilA protein. To cleave the fusion protein with this method, the TEV protease was produced with a yield of approximately 1 mg/L of cell culture. However, attempts to cleave the fusion protein with the TEV protease were unsuccessful, despite being tested under several conditions (Triton X-100, DDM, β -OG and DHPC detergents). Since, while these expression assays were being tested, it was discovered that pili was not directly involved in the EET process, no further optimizations were made regarding the expression and purification of PilA protein.

5.5 References

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MOLECULAR INTERACTIONS STUDIED BY NUCLEAR MAGNETIC RESONANCE¹

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6. Molecular interactions studied by Nuclear Magnetic Resonance

6.1 Biomolecular interaction studies between cytochrome PpcA from *Geobacter sulfurreducens* and the electron acceptor ferric nitrilotriacetate (Fe-NTA)

As previously mentioned, the conceptual model for the extracellular electron transfer in *G. sulfurreducens* considers that different proteins are involved in oxidation of the inner membrane quinols depending upon the redox potential of the final electron acceptor: the CbcL-dependent pathway operates at or below redox potentials of -100 mV (relative to the NHE), whereas the ImcH-dependent pathway operates above this redox potential [1, 2]. In each case, electrons are supplied from the quinol oxidases to the PpcA-family cytochromes. The electrons are then transferred from periplasmatic to outer membrane associated cytochromes, through porin-cytochrome trans-outer membrane complexes toward extracellular acceptors. Whatever is the active respiratory pathway, the periplasmic cytochromes are always present and particularly the cytochrome PpcA, which is the most abundant cytochrome in *G. sulfurreducens* [3, 4]. It is the most likely reservoir of electrons destined for outer surface [5, 6] and it may also contribute to the periplasmic electron storing if external acceptors are temporary unavailable [7, 8]. Interacting interface regions between this cytochrome and electron acceptors were recently characterized, using a set of complementary biophysical techniques including stopped-flow kinetics, molecular docking, UV-visible and NMR measurements. These studies allowed to identify the molecular complexes established between cytochrome PpcA and anthraquinone-2,6-disulfonate (AQDS), anthrahydroquinone-2,6,-disulfonate (AH₂QDS) and Fe(III)-citrate [9-11]. The results obtained also suggested that the cytochrome PpcA may function as terminal reductase for AQDS and Fe(III)-citrate fractional populations that are able to transverse the outer membrane and reach the periplasm. However, genetic and proteomic studies showed that the reduction of the humic substance analog (AQDS) and Fe(III)-citrate by *G. sulfurreducens* and *Shewanella oneidensis* cells is essentially carried out by the outer membrane cytochromes [12-15], suggesting that the direct reduction of these acceptors by PpcA is not significant.

Ferric nitrilotriacetate (Fe-NTA) can also be utilized by *G. sulfurreducens* and *S. oneidensis* as electron acceptor [16]. Fe-NTA is found in several natural environments, particularly in sewage or contaminated waters [17]. Chelators, such as NTA, have an important role in the solubilization of Fe(III) and concomitant enhancement of its bioavailability [18]. The stimulation of Fe(III) reduction may be used in several bioremediation applications, including the contaminant degradation of aromatic hydrocarbons in sediments from petroleum-contaminated aquifers [19]. This iron chelated form is smaller and more outer-membrane permeable compared to Fe(III)-citrate [12]. Therefore, it is expected that periplasmic reduction

of Fe-NTA would be considerably higher compared to Fe(III)-citrate. This was in fact confirmed by Gescher [13] using a synthetic biology approach involving the reconstruction of the putative electron transport chain of the bacterium *S. oneidensis* in *Escherichia coli*. In addition, it was also shown that *S. oneidensis* outer membrane cytochromes contribute to the global reduction of Fe-NTA [20]. Thus, given the cellular architecture similarity between *S. oneidensis* and *G. sulfurreducens*, it is conceivable that, in addition to periplasmic cytochromes, *G. sulfurreducens* outer membrane cytochromes also contribute to the reduction of Fe-NTA.

To provide evidence that PpcA can also function as terminal reductase and further contribute to the understanding of soluble iron respiratory pathways in *G. sulfurreducens*, we used UV-visible and NMR spectroscopic techniques to investigate the biomolecular interactions between PpcA and Fe-NTA and to map the interface interacting regions in the redox complex. The results obtained allowed us to identify a specific region of interaction between the two molecules. Overall, the information obtained provides important foundations to the understanding of the iron respiratory pathways in *G. sulfurreducens* and can be explored for optimal bioelectrochemical applications.

6.1.1 Materials and Methods

Natural abundance and ^{15}N -isotopic labeled samples of cytochrome PpcA were expressed and purified as previously described for cytochrome PpcF in section 4.2.2.

6.1.1.1 UV-visible experiments

All the solutions and manipulations were performed at 25 °C under strict anaerobic conditions (oxygen levels kept under 1 ppm), inside a glove box chamber (MBraun LABstar) with argon circulation to avoid sample reoxidation. Electronic absorption spectra were acquired in the range 350–600 nm, using an Evolution 300 UV-vis spectrophotometer (Thermo Fisher Scientific) equipped with a Fiber Optic Coupler and fiber optics inside the anaerobic chamber.

Protein samples (3 μM) were prepared in phosphate buffer pH 7.0 with NaCl (100 mM final ionic strength). A concentrated Fe-NTA solution (3 mM) was prepared in the same buffer, accordingly to the procedure described by Bunescu [21]. Reduction of PpcA was obtained by adding a stoichiometric amount of sodium dithionite.

6.1.1.2 NMR studies

For NMR studies, samples of PpcA were prepared in phosphate buffer pH 7.0 with NaCl (100 mM final ionic strength) in 92% H_2O /8% $^2\text{H}_2\text{O}$ (^{15}N -labeled sample) or pure $^2\text{H}_2\text{O}$ (unlabeled sample). All the NMR experiments were performed in a Bruker Avance 600 MHz spectrometer at 25 °C. The ^1H chemical shifts are reference to DSS at 0 ppm, by using the residual water signal as an internal secondary reference [22] and the ^{15}N shifts were calibrated through indirect referencing [23].

Data were processed using TOPSPIN software (Bruker Biospin, Karlsruhe, Germany) and analyzed with the program Sparky (TD Goddard and DG Kneller, Sparky 3, University of California, San Francisco, United States). The following set of experiments was acquired: (i) 2D ^1H , ^{15}N -HSQC for the ^{15}N labeled sample in 92% H_2O / 8% $^2\text{H}_2\text{O}$, and (ii) 2D ^1H , ^{13}C -HSQC; 2D ^1H -NOESY with 80 ms mixing-time and 1D ^1H NMR spectra in pure $^2\text{H}_2\text{O}$. The backbone NH and heme methyl signals of PpcA were previously assigned [10, 24, 25] and were used to monitor the effect of the addition of Fe-NTA. The effect of Fe-NTA addition on the heme signals was monitored by the analysis of a series of 1D ^1H NMR spectra. This effect was also studied via 2D ^1H , ^{13}C -HMQC and 2D ^1H -NOESY obtained in the absence and presence of PpcA (1:3 PpcA:Fe-NTA ratio). The effect of the addition of Fe-NTA in the PpcA cytochrome backbone NH signals was monitored by recording 2D ^1H , ^{15}N -HSQC NMR spectra in the absence and presence of PpcA (1:3 PpcA:Fe-NTA ratio). The pH of the samples was measured before and after each NMR spectra series. To confirm protein integrity 1D ^1H NMR spectra were acquired before and after each 2D spectrum.

Finally, to investigate the binding reversibility, 1D ^1H NMR spectra were acquired for free PpcA, 1:3 PpcA:Fe-NTA ratio and after removal of Fe-NTA by ultrafiltration using Amicon Ultra devices with a 3 kDa molecular weight cutoff.

6.1.2 Results and discussion

6.1.2.1 Probing the reduction of Fe-NTA with cytochrome PpcA by UV-visible spectroscopy

The different UV-visible absorption spectral features of cytochrome PpcA in the reduced and oxidized forms were explored to study the reduction of Fe-NTA (Figure 6.1). In fact, the UV-visible spectrum of PpcA in the oxidized state showed a peak at 406 nm (Soret band) and a very broad peak in the range 510–570 nm. On the other hand, three distinct bands are clearly visible in the reduced form: 417 nm (Soret band), 522 nm (β band), and 552 nm (α band). Thus, we monitored the effect of the addition of a Fe-NTA solution to a reduced PpcA sample. With the concomitant addition of Fe-NTA the characteristic bands of the reduced cytochrome disappear and are replaced by those of the oxidized form, confirming that PpcA is able to reduce Fe-NTA (Figure 6.1).

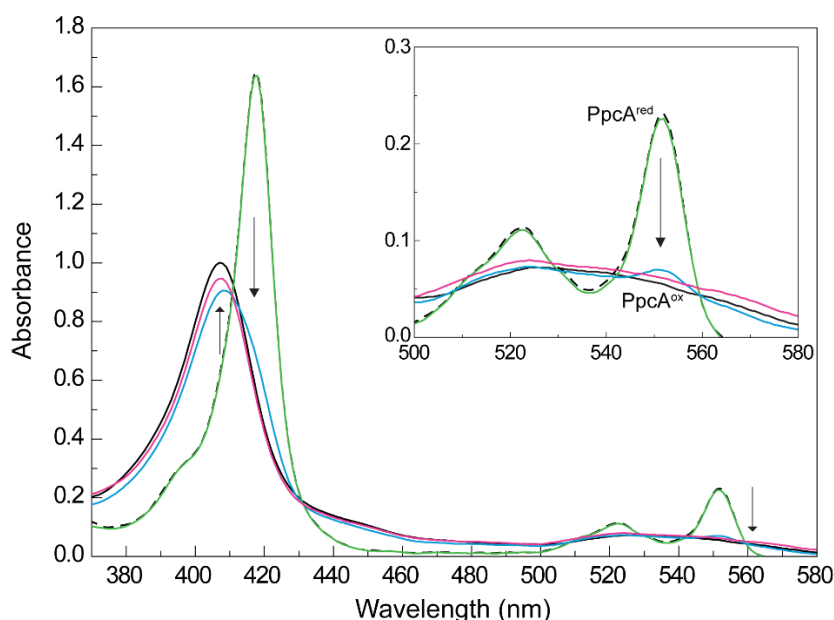


Figure 6.1 - PpcA oxidation by Fe-NTA monitored by UV-visible spectroscopy at 25 °C and pH 7. The UV-visible spectra of PpcA in the oxidized (PpcA^{ox}) and reduced (PpcA^{red}) forms are indicated by the continuous and dashed black lines, respectively. The concentration of PpcA used in the experiments was 3 μ M. Spectra obtained for PpcA:Fe-NTA ratios are indicated by colored lines: 1:1 (green), 1:3 (blue), and 1:10 (pink). The arrow points to increasing concentrations of Fe-NTA. For clarity, an enlarged scale of the spectra between 500 and 580 nm is showed in the inset.

Compared to other iron chelated forms, the equilibrium speciation in solution of Fe-NTA at pH 7.0 is less complex and essentially one main iron form is present: $[\text{FeOH-NTA}]^-$ (92%) versus $[\text{Fe}(\text{OH})_2\text{-NTA}]^{2-}$ (8%) [20]. The higher reduction potential of the dominant iron form, +0.372 mV, [26] compared to those of PpcA heme groups: -147, -104, and -111 mV for hemes I, III, and IV, respectively [27] explains the favorable reduction of FeOH-NTA^- by the cytochrome. The negative reduction potential of PpcA heme groups indicates that the electron acceptor can be reduced by any of them. To elucidate if one or more heme groups are directly involved in the reduction of Fe-NTA, we then studied the molecular interactions between the two molecules by NMR.

6.1.2.2 NMR interaction studies between PpcA and Fe-NTA probed by NMR

Nuclear Magnetic Resonance chemical shift perturbation experiments are extremely powerful to identify protein-protein or protein-ligand complexes by providing wealthy structural information about the nature of the complex interacting interfaces. Theoretically, NMR chemical shift perturbation experiments can be monitored by any type of NMR spectrum. However, the 2D $^1\text{H},^{15}\text{N}$ -HSQC NMR spectra are the most popular ones. In fact, these experiments correlate the chemical shifts of the nitrogen nuclei, in a ^{15}N -labeled protein, with their directly attached protons. Thus, as every amino acid contains a peptide bond NH group, except proline residues, 2D $^1\text{H},^{15}\text{N}$ -HSQC NMR experiments obtained for the cytochrome sample in the absence and presence of its putative partner(s) will provide a good fingerprint of the binding interface. Indeed, upon complex formation the chemical environment of the interacting residues will be affected and, as a consequence, their NMR signals. In the case of a protein-ligand complex, these changes can be reported by chemical shift variations of the affected residues and/or by the line width broadenings. The latter is observed in case of paramagnetic ligands whose unpaired electrons decrease the relaxation rates and increase the line widths of interacting groups in the protein (for a review see [28]).

However, in the case of cytochromes, the study of molecular interactions is further complex due to the substantial differences in the NMR spectra of the diamagnetic and paramagnetic forms. Indeed, in the oxidized state, the paramagnetic effect of the unpaired electron(s) at the heme iron spreads the heme substituent NMR signals and neighboring residues over larger spectral widths, compared to the reduced and diamagnetic state. In fact, in the particular case of cytochrome PpcA from *G. sulfurreducens*, the protein signals are spread over a spectral window between -5 to 21 ppm in the oxidized state, whereas in the reduced state they cover a substantial smaller spectral window (-2 to 11 ppm) [29]. Therefore, the study of the interacting regions between PpcA and its putative redox partners cannot be accomplished by the addition of increasing amounts of the electron acceptor in the oxidized state to PpcA in the reduced form. If this was the case, the NMR spectral changes due to the

formation of the redox complex will be masked by the more significant redox-linked signal spectral changes. For this reason, the study of the interacting regions in redox partners is carried out by keeping them in the same oxidation state [30-33].

The solution structures of PpcA have been determined in the fully oxidized and fully reduced forms [34, 35]. The structures are highly conserved suggesting that the interacting regions with putative redox partners can be mapped in experiments carried out with the protein in the reduced or oxidized state. This was indeed confirmed by NMR chemical shift perturbation studies between PpcA and the humic substance analog, which revealed that this molecule interacts in the same region with the cytochrome [9, 10].

From the above, and given the smaller NMR signal overlap in the oxidized state, the molecular interactions between PpcA and the electron acceptor Fe-NTA were investigated in this oxidation state. 2D ^1H , ^{15}N -HSQC experiments were used to probe the chemical shift perturbations at the PpcA polypeptide backbone NH signals. These signals have been previously assigned [10]. All signals were assigned except those of Ala¹ and Asp² due to fast exchange with the solvent; Gly³⁶ and Gly⁴² due to signal broadening caused by the nearby hemes paramagnetic irons and Cys⁵¹ due to its rapid solvent exchange.

The effect of the addition of Fe-NTA on PpcA NH signals is illustrated in Figure 6.2. The comparison of the 2D ^1H , ^{15}N -HSQC NMR spectra acquired in the absence and presence of Fe-NTA indicates that the free and bound structures of PpcA are similar on side chains position since the spectral dispersion of the NMR signals is maintained (Figure 6.2; see also Figure 6.3 and Figure 6.4). However, important signal line width broadening as well as chemical shift variation though at a lesser extent, was observed upon addition of Fe-NTA. The increase in the line width broadening, as a function of Fe-NTA concentration, is explained by the paramagnetic high spin ($S=5/2$) character of the ferric iron [36] and concomitant increase in the relaxation rates of the neighboring nuclei.

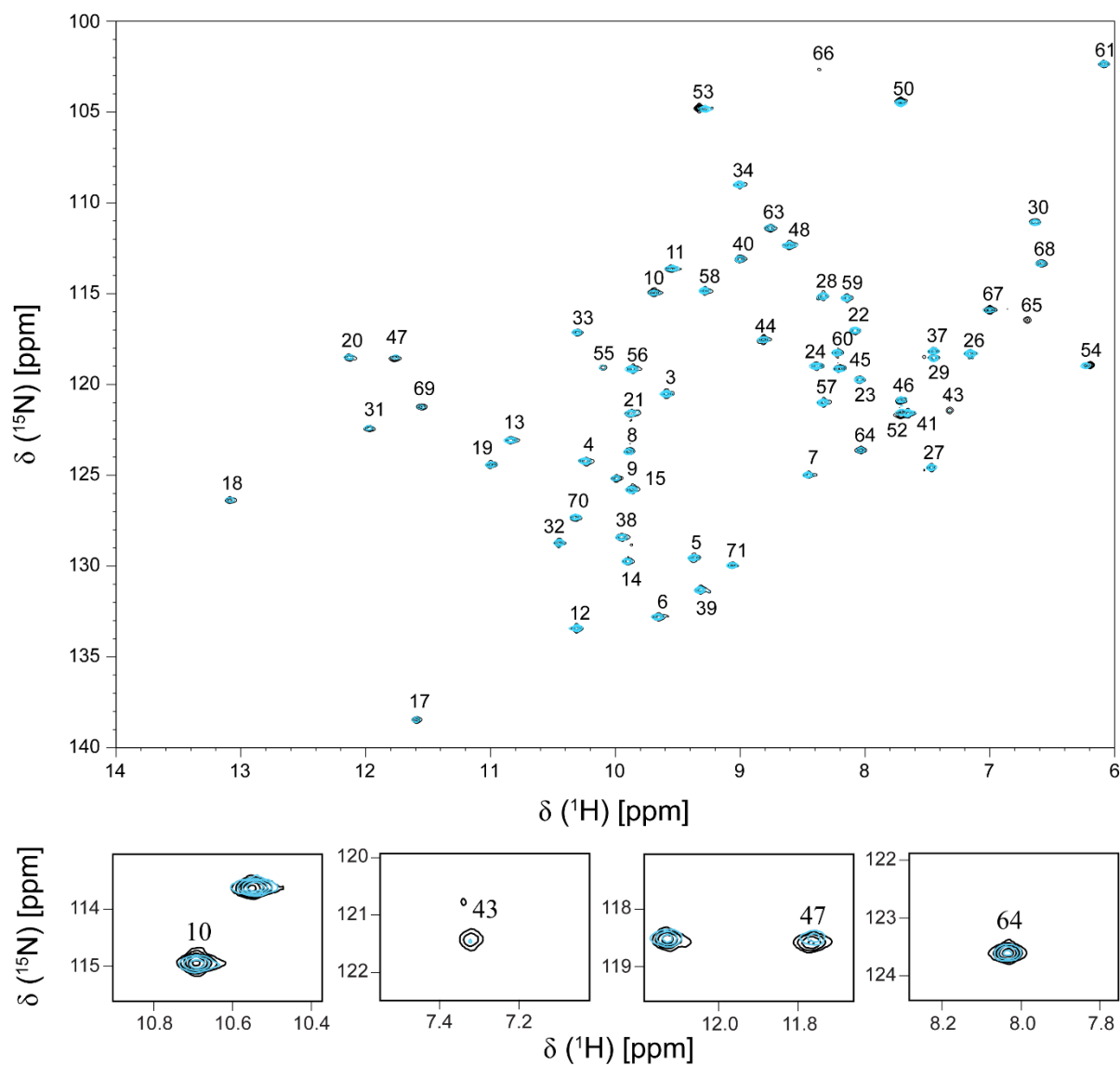


Figure 6.2 - Overlay of the 2D ^1H , ^{15}N -HSQC NMR spectra of ^{15}N -enriched PpcA sample acquired in the absence (black) and in the presence (cyan) of Fe-NTA. The spectrum in presence of Fe-NTA was obtained for a PpcA:Fe-NTA ratio of 1:3. The assignment of the NH signals is indicated. For clarity, expansions of the most affected signals are shown at the bottom of the figure.

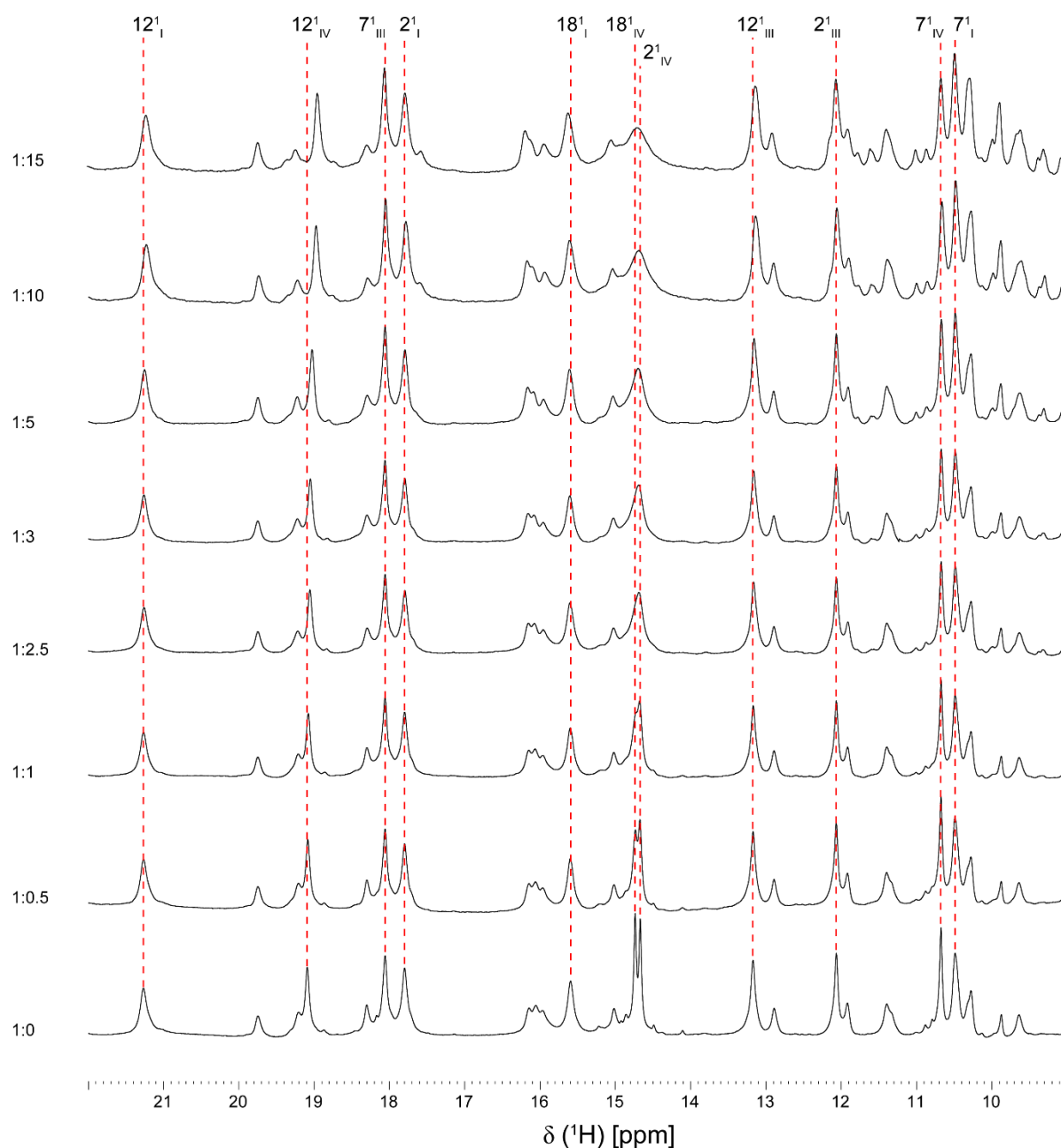


Figure 6.3 - Expansions of the low-field region of 1D ^1H NMR spectra obtained for PpcA in the presence of increasing amounts of Fe-NTA (pH 7 and T 25 °C). The heme methyls labeled accordingly to the IUPAC nomenclature for tetrapyrroles [37] are indicated, with exception of the $18^{\text{I}}\text{CH}_3^{\text{III}}$ signal which appears at a chemical shift of 0.76 ppm.

As shown by the UV-visible experiments, Fe-NTA can be reduced by the cytochrome PpcA. Therefore, to assure an effective electron transfer within the redox complex, at least one heme group must be in the vicinity of the acceptor. However, due to the similar reduction potential values of PpcA heme groups, the electron acceptor can be putatively reduced by any heme. As a consequence of the small size of cytochrome PpcA, which displays a ratio of 24 residues per heme group, it is expected that any perturbation caused by binding of the Fe-NTA would affect the heme substituent NMR signals. The heme methyl group signals are

found in less crowded regions of the NMR spectra and therefore are the best heme substituents to monitor this effect (see Figure 6.3). All the heme methyl signals of PpcA were previously assigned [25] and both 1D ^1H and 2D ^1H -NOESY NMR spectra were used to further evaluate the molecular interactions between Fe-NTA and PpcA. Figure 6.3, indicates the region of 1D ^1H NMR spectra that contains the heme methyl signals, except $18^1\text{CH}_3^{\text{III}}$, which appears at 0.76 ppm. Since this signal cannot be followed by 1D ^1H NMR titrations, the evaluation of the addition of Fe-NTA on the full set of heme methyl signals was monitored by 2D ^1H , ^{13}C -HMQC NMR spectra recorded in the absence and presence of the electron acceptor (Figure 6.4A). The analysis of the referred spectra confirms that the protein conformation is conserved in the presence of Fe-NTA since the chemical shift dispersion of the signals is maintained. In addition, there is a significant line width broadening of the heme IV methyl signals. The heme methyls $2^1\text{CH}_3^{\text{IV}}$ and $18^1\text{CH}_3^{\text{IV}}$ are clearly the most affected ones compared to $7^1\text{CH}_3^{\text{IV}}$ and $12^1\text{CH}_3^{\text{IV}}$ signals. These observations were further confirmed by an analysis of the heme methyl NOE connectivities (Figure 6.4B).

In order to assure directionality in the reduction of the electron acceptor it is important that the interaction is reversible. This was evaluated by the comparison of 1D ^1H NMR spectra of PpcA recorded before and after the removal of the electron acceptor by ultrafiltration, which confirms that the interaction is fully reversible (Figure 6.5).

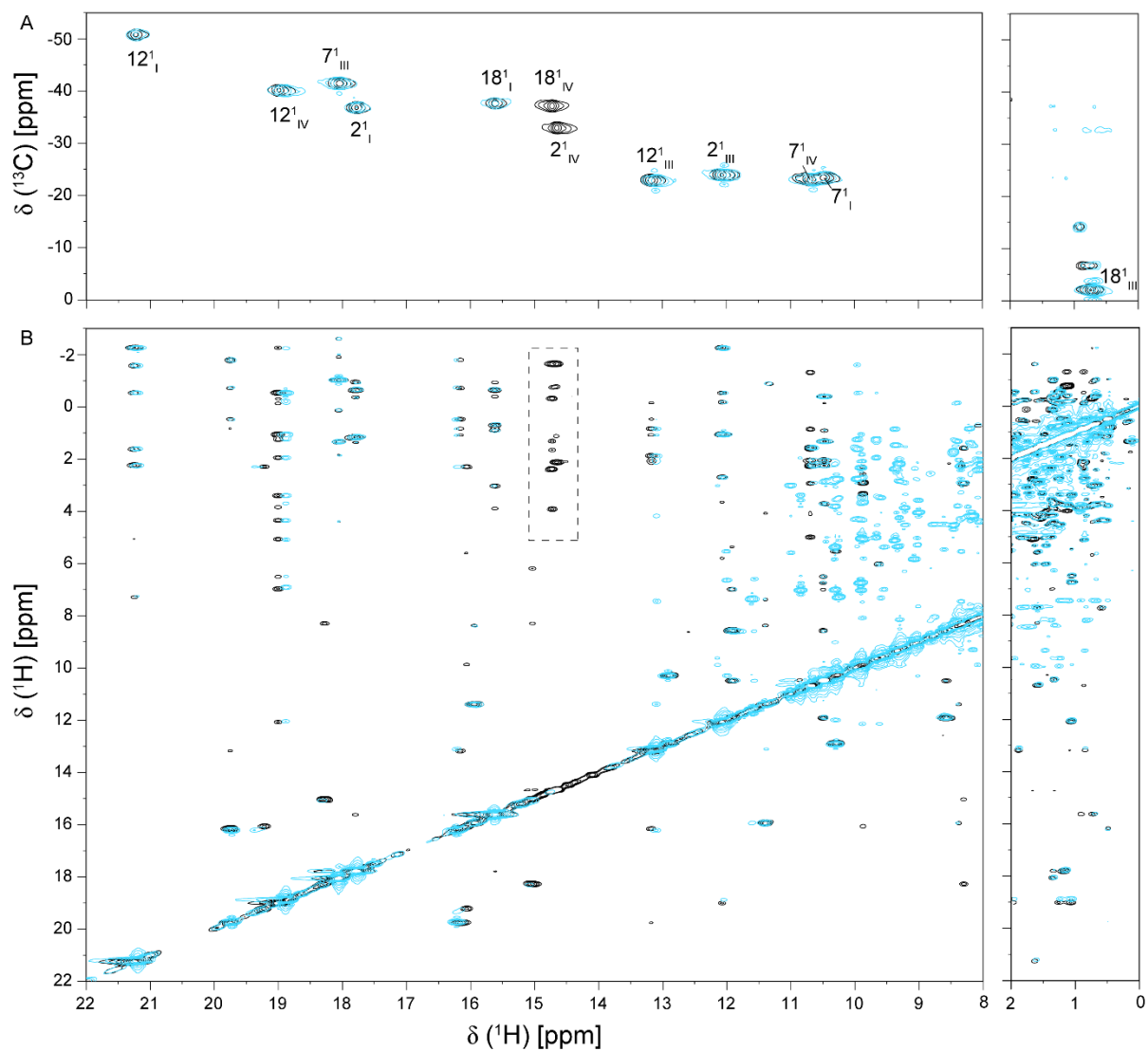


Figure 6.4 - Selected regions of 2D ^1H , ^{13}C -HMQC (A) and 2D ^1H -NOESY (B) NMR spectra of cytochrome PpcA in the absence (black) and presence (cyan) of Fe-NTA (pH 7 and 25 °C). The dashed rectangle shows the NOE connectivities of heme methyls $2^{\text{I}}\text{CH}_3^{\text{IV}}$ and $18^{\text{I}}\text{CH}_3^{\text{IV}}$, which presents significant broadening in the presence of Fe-NTA.

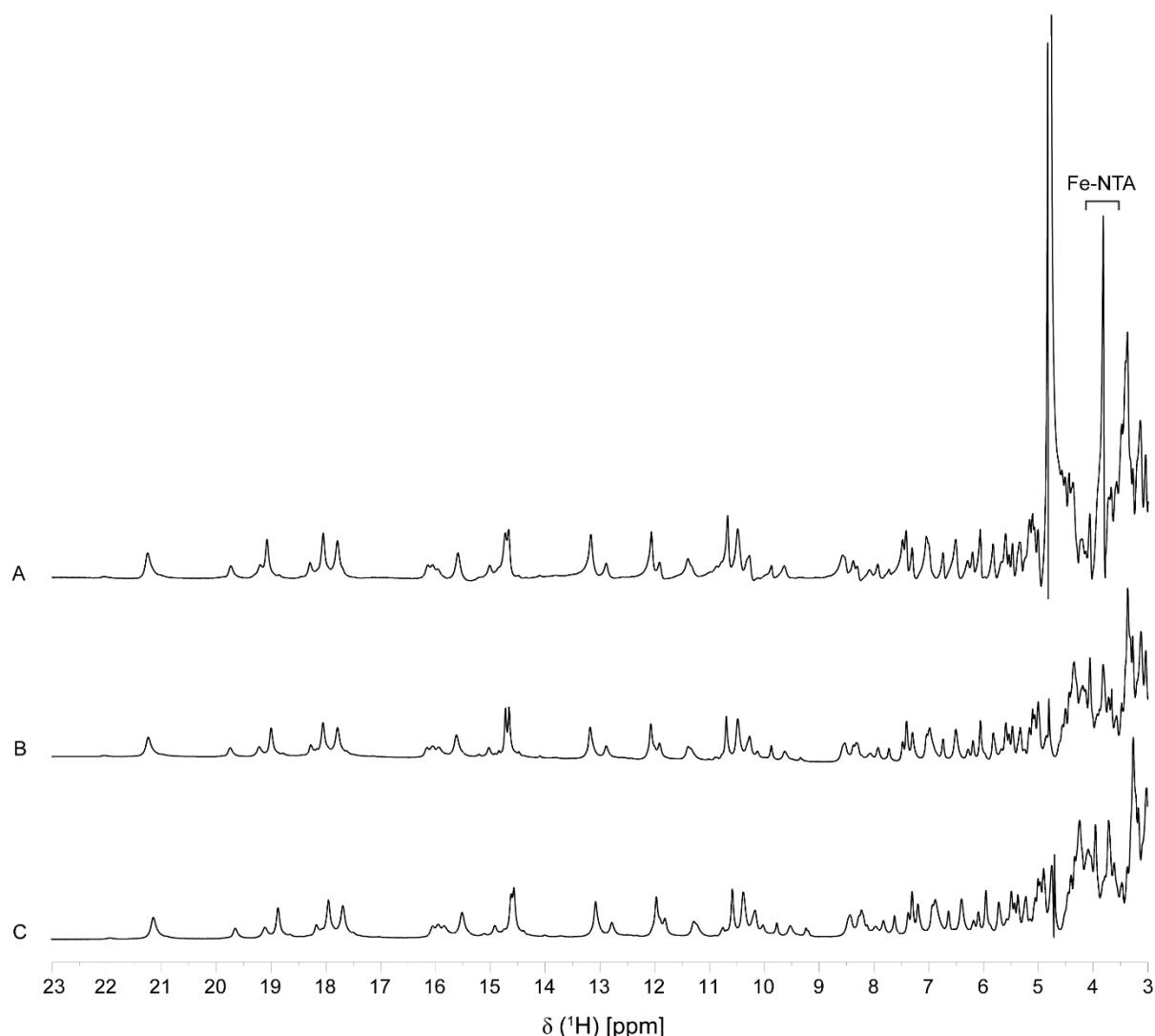


Figure 6.5 - Reversibility of Fe-NTA binding to PpcA monitored by 1D ^1H NMR (pH 7 and 25 °C). A fingerprint region of the PpcA 1D ^1H NMR spectrum is shown: (A) PpcA in the presence of Fe-NTA (PpcA:Fe-NTA ratio 1:0.5); (B) PpcA prior to the addition of Fe-NTA, and (C) PpcA after removal of Fe-NTA.

6.1.2.3 Structural rationale for the interface interacting region between PpcA and Fe-NTA

The residues whose NH backbone signals showed a decrease in the peak data height above 40% are Lys⁹, Asn¹⁰, Lys¹⁸, Lys⁴³, Glu⁴⁴, His⁴⁷, Lys⁶⁴, Cys⁶⁵, Gly⁶⁶, Glu⁶⁷, Cys⁶⁸, His⁶⁹, and Lys⁷¹ (Figure 6.6A). The most affected residues included the PpcA heme IV binding motif (Cys⁶⁵-Gly⁶⁶-Glu⁶⁷-Cys⁶⁸-His⁶⁹) and, except for Lys¹⁸, all the others are located near this heme (Figure 6.6B). A small set of sequential residues (Lys⁵², Gly⁵³, and Cys⁵⁴) experienced a considerably smaller line width broadening (Figure 6.6A) but chemical shift perturbation was observed in their NH signals (see also Figure 6.2). These three residues were also mapped on the structure of PpcA and their proximity to heme IV increases from Lys⁵² to Cys⁵⁴ (see blue colored residues in Figure 6.6B). Therefore, the observed effect probably reflects the propagation of

perturbations from the binding surface closer to heme IV (see red colored residues in Figure 6.6B) rather than direct binding.

The effect of the addition of Fe-NTA on PpcA monitored by the heme methyl signals also confirms the interaction between the two molecules in the vicinity of heme IV. In fact, the most solvent exposed heme methyls ($2^1\text{CH}_3^{\text{IV}}$ and $18^1\text{CH}_3^{\text{IV}}$) are clearly the most affected ones (see Figure 6.6C).

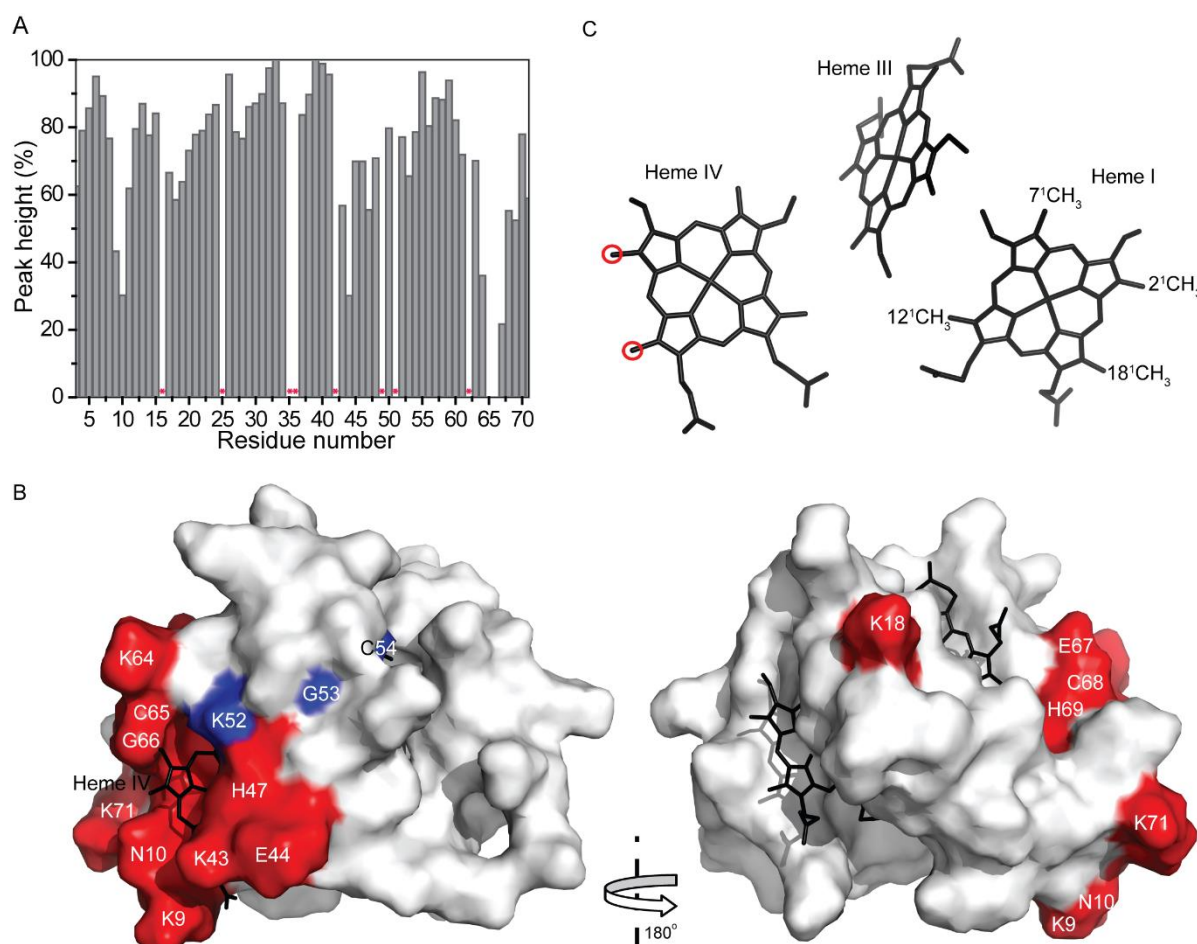


Figure 6.6 - Line width broadening and chemical shift perturbations on PpcA from *Geobacter sulfurreducens* NMR signals. (A) Variation of the peak height of the PpcA signals (%) measured from the 2D ^1H , ^{15}N -HSQC NMR spectra indicated in Figure 6.2. Red asterisks indicate proline or non-assigned residues. (B) Surface mapping of the most affected residues. The red and blue residues correspond to those that showed a decrease in the peak data height above 40% and a significant deviation in their chemical shift, respectively. The heme groups are represented in black. The molecular surface was generated in Pymol [38] using the structure of PpcA [PDB code: 2LDO [35]]. The left and right panels are related by a 180° rotation. (C) Heme core of PpcA [PDB code: 2LDO [35]]. Red circles indicate the most affected heme methyl signals (see also Figure 6.3).

		10		20		30		40		50		60		70																																																									
PpcA:Fe-NTA	A	D	D	I	V	L	K	A	K	N	G	D	V	K	F	P	H	K	A	H	Q	K	A	V	P	D	C	K	K	C	H	E	K	G	P	G	K	I	E	G	F	G	K	E	M	A	H	G	K	G	C	K	G	C	H	E	E	M	K	K	G	P	T	K	C	G	E	C	H	K	K
PpcA:Fe-Citrate	A	D	D	I	V	L	K	A	K	N	G	D	V	K	F	P	H	K	A	H	Q	K	A	V	P	D	C	K	K	C	H	E	K	G	P	G	K	I	E	G	F	G	K	E	M	A	H	G	K	G	C	K	G	C	H	E	E	M	K	K	G	P	T	K	C	G	E	C	H	K	K
PpcA:A(H₂)QDS	A	D	D	I	V	L	K	A	K	N	G	D	V	K	F	P	H	K	A	H	Q	K	A	V	P	D	C	K	K	C	H	E	K	G	P	G	K	I	E	G	F	G	K	E	M	A	H	G	K	G	C	K	G	C	H	E	E	M	K	K	G	P	T	K	C	G	E	C	H	K	K

Figure 6.7 - Comparison of the most affected residues in the redox complexes established between PpcA from *G. sulfurreducens* and the electron acceptors Fe-NTA (this work), Fe-citrate [11] and AQ(H₂)DS [9, 10]. The residues highlighted in red and blue correspond to those that showed a decrease in the peak data height above 40% or a significant deviation in their chemical shift, respectively.

6.1.3 Conclusions and implications

In the present work, we showed that Fe-NTA can be directly reduced by the triheme cytochrome PpcA from *G. sulfurreducens*. The results obtained are in accordance with the reduction potential values of both molecules at pH 7. The reduction potential values of the three heme groups of PpcA cover the functional range -147 to -104 mV [27], whereas that of Fe-NTA is considerably higher ($+0.372$ mV), favoring the thermodynamic reduction of the latter. Since any of the PpcA heme groups can hypothetically reduce the electron acceptor, we also probed the molecular interaction between the cytochrome and the Fe-NTA by NMR. The results showed that the cytochrome interaction region comprises a high number of positively charged lysine residues located in the vicinity of the most exposed edge of heme IV. However, the other two hemes are also considerably exposed to the solvent [35, 39], which anticipates that the interaction between the Fe-NTA molecule and PpcA heme IV is directed by other factors. Considering the negative charge of the most abundant form of the Fe-NTA in solution at pH 7.0 (FeOH-NTA⁻), electrostatic interactions driving by the positively charged lysine residues in the vicinity of heme IV are expected to favor the formation of the PpcA:FeOH-NTA⁻ redox complex.

Interestingly, previous studies have also shown that the heme IV region in the cytochrome PpcA from *G. sulfurreducens* was also involved in the formation of redox complexes with other electrons acceptors, namely the humic substance analog (AQDS) and Fe-citrate [10, 11]. A comparison of the interacting polypeptide regions between PpcA and the three electron acceptors are summarized in Figure 6.7. Just as the dominant species of Fe-NTA at pH 7.0, the charge of these two electron acceptors is also negative. Therefore, the available data for the molecular interacting regions between PpcA and different negatively charged *G. sulfurreducens* terminal electron acceptors unveils the structural and functional features underlying the effective formation of the redox complexes and concomitant reduction of the different electron acceptors. Indeed, in order to efficiently reduce the electron acceptor, the heme directly involved in this reduction must have (i) a lower reduction potential value compared to those of the electron acceptors and (ii) a positively charged surface in its vicinity to electrostatically assist in the redox complex formation. Thus, these two observations can putatively be used as starting models to assist the optimization of the molecular interaction between cytochromes that could interact with negatively charged electron acceptors by enhancing their reactivity and to improve biotechnological applications with engineered *Geobacter* strains with optimal desirable properties for the bioremediation of environmental organic and metal contaminants [40]. In addition, the ability of cytochrome PpcA to reduce toxic and radioactive compounds [41] coupled to its robustness, has been shown by its high stability ($T_m > 85$ °C) [42] and maintenance of its mechanistic and functional features over a wide range of pH and ionic strength values [43, 44], pave the way to develop rational PpcA based bioelectrochemical strategies to the bioremediation of toxic/radioactive metals.

6.2 Periplasmic electron transfer network in *Geobacter sulfurreducens* revealed by biomolecular interaction studies

The co-existence of highly structurally homologous electron transfer proteins sharing the same cellular compartment is intriguing, in the light of the formation of specific complexes expected to be active in distinct respiratory pathways. This feature seems to be a hallmark of the electrogenic *Geobacter* bacteria. In fact, *Geobacter* cells contain numerous multiheme cytochromes that participate in electron transfer pathways involving the reduction of distinct extracellular electron acceptors, including iron oxides, manganese oxides or the surface of electrodes [45]. Periplasmic triheme cytochrome families are particularly abundant in these bacteria and crucial to efficiently bridge the electron transfer resulting from the oxidation of cytoplasmic compounds with the cell exterior. Amongst the various *Geobacter* species, the periplasmic triheme family from *Geobacter sulfurreducens*, the so-called PpcA-family, is the best characterized. Their crystal [39, 42, 46] and NMR [34, 35] structures showed that the five cytochromes have the lowest and the highest structural similarity near hemes placed at the N- and C-terminal region, respectively. Concerning the heme iron atom of the hemes, as previously mentioned, they are in the low-spin state both in the reduced (diamagnetic, $S=0$) and oxidized (paramagnetic, $S=1/2$) states yielding well-resolved and distinct NMR spectra (Figure 8.1, Appendix). These features have been explored to assign the backbone NMR fingerprints (except for PpcC) and the heme signals in the reduced and oxidized states [10, 11, 24, 25, 47, 48]. The functional properties of each cytochrome were also characterized in detailed, including the determination of the individual heme reduction potential values (except for PpcC due to the presence of multiple conformations in solution) [4, 46]. Despite the high sequence and structure homology of the cytochromes, their functional properties are quite distinct, suggesting likewise a specific role in the cell.

Complementary proteomic, whole-genome microarrays and gene deletion studies in the presence of different electron acceptors were carried out for the members of this cytochrome family [3, 5, 49-51]. The studies showed that in the presence of different electron acceptors (Fe(III) citrate, Fe(III) or Mn(IV) oxides), some of the cytochromes are overexpressed or upregulated, but the presence of distinct members of the family seems to be constitutive. Moreover, the more recent results suggest that in the mutants lacking four of the homologs, the remaining homolog present in the periplasm is able to support the reduction of the terminal electron acceptor and contributes to the adaptation of the strains [52]. Additionally, quintuple mutants were still able to reduce Fe(III) and this fact was explained by the presence of an unrelated triheme cytochrome (PgcA), as well as other extracellular cytochromes as OmcE and OmcS that can contribute to electron transfer in the periplasm [52]. Interestingly,

when electrodes were used as terminal acceptors, the growth rate of the quintuple mutant was virtually unaffected.

Given the constitutive presence of the periplasmic triheme cytochrome pool in *G. sulfurreducens*, we explored the distinct NMR fingerprints of each protein to investigate the molecular interactions and the electron transfer reactions between each pair of cytochromes. The pattern of reactivities shows that all proteins interact transiently, while exchanging electrons promiscuously until they reach an intermediate oxidation state. The putative advantages for the bacterium of having such pool of complementary cytochromes are discussed.

6.2.1 Materials and Methods

6.2.1.1 Protein expression and purification

The five triheme cytochromes (PpcA-E) were overexpressed in *E. coli* and purified according to the protocols previously described [42, 53, 54]. After purification, cytochromes were buffer exchanged to 8 mM sodium phosphate buffer pH 7.4 with NaCl (final ionic strength 20 mM). PpcA, PpcB, PpcD and PpcE were concentrated up to 1-1.5mM, while PpcC could only be concentrated up to ~200 μ M in the experimental conditions.

6.2.1.2 Sample preparation for NMR studies

All cytochromes' samples (final concentration 80 μ M and 3.2 mM for interactions in the oxidized state and 50 μ M and 500 μ M for electron transfer experiments) were prepared in 8 mM sodium phosphate buffer pH 7.4 with NaCl (final ionic strength 20 mM) and pure $^2\text{H}_2\text{O}$ (99% atom, CIL isotopes) after two cycles of lyophilization, which were previously shown not to affect the proteins' integrity [4, 46].

6.2.1.3 Probing biomolecular interactions and electron transfer reactions with NMR

NMR spectra were acquired at 25 °C in a Bruker Avance III 600 MHz spectrometer equipped with a triple-resonance cryoprobe (TCI). ^1H chemical shifts were calibrated using 2,2-dimethyl-2-silapentane-5- sulphonate as reference at 0 ppm [22] and TOPSPIN software (Bruker Biospin, Karlsruhe, Germany) was used for spectra processing and analysis.

Molecular interactions between each pair of triheme cytochromes were followed by 1D ^1H NMR chemical shift perturbation experiments. The titrations were performed in the oxidized state up to 1:5 or 1:10 protein ratios, by adding increasing amounts of a concentrated sample of one cytochrome (3.2 mM) to an 80 μ M sample of another cytochrome. The experiments were performed for every cytochrome (PpcA-E) in the presence of each other in both directions, in a total of 20 experiments (e.g. PpcA titrated with the other four homologs,

and so on). 1D ^1H NMR spectra were acquired after each addition, with a sweep width of 50 kHz and 512 scans.

To monitor electron transfer between the cytochromes, each protein was prepared at 50 μM and sealed with a gas-tight serum cap in an NMR tube. The samples were reduced by flushing hydrogen in the tube in the presence of catalytic amounts of hydrogenase and a 1D ^1H NMR spectrum was acquired in the reduced state. Hydrogen was then exchanged to argon before the addition of each putative oxidized partner in a 1:1 ratio inside a glovebox. A 1D ^1H NMR spectrum was acquired, and after the tube was opened for the acquisition of another spectrum with both proteins oxidized. 20 experiments were performed to account for all the combinations.

6.2.2 Results and discussion

PpcA-family members are involved in pathways where the electron acceptor is whether Fe(III) citrate, Fe(III) or Mn(IV) oxides, which indicates that in many growing conditions they are co-expressed. Hence, the possibility of interaction between these cytochromes was probed by NMR chemical shifts perturbation experiments. These are experiments that are widely used to identify protein–protein or protein-ligand complexes. Indeed, while in an interacting protein pair the signals of the groups in the interacting interface will have a different chemical environment and concomitantly will show a variation in their chemical shift, compared to those of the isolated forms, in the absence of an interaction the NMR spectrum remains unaltered. Hence, this type of experiment provides structural information about the nature of the interacting interfaces in the complex. In the present work, 1D ^1H NMR spectra chemical shift perturbation experiments were carried out for the different combination of cytochrome pairs by adding successive amounts of one cytochrome to the other and vice-versa in the oxidized state. In the particular case of redox proteins it is necessary to take into account that their NMR spectra are quite distinct in the reduced and oxidized states (Figure 8.1, Appendix) and hence to map the putative interacting regions between the different cytochrome by these type of experiments, these must be conducted by keeping the target molecules in the same oxidation state so that any change can be attributed to an interaction and not to a variation in the oxidation state. The choice of the oxidized state over the reduced state has several experimental advantages. Firstly, given the low redox potential values of the cytochromes under study (Table 6.1), anaerobic manipulation of the samples is not required. Secondly, in the oxidized state the heme signals with the highest intensity (heme methyl groups) are very well separated from the envelope of the protein signals. Thirdly, the NMR spectra of the five cytochromes are more distinct in the oxidized state, which permits the monitorization of the chemical shift variations throughout a titration more accurately (Figure 8.1, Appendix). The NMR spectra obtained for the chemical shift perturbation experiments are shown in Figures 8.2-8.6 (Appendix). Experimental conditions at low ionic strength were selected to favor

electrostatic interactions that are typically observed in redox complexes. In these conditions, and as stated in the Methods, PpcC could only be concentrated to lower concentrations when compared with the other cytochromes due to precipitation. This drawback influenced the titration results when PpcC was titrated on the other cytochromes, since its addition led to a large variation of the ionic strength on the NMR tube (Figure 8.7, Appendix). Thus, the results observed can be due to changes in ionic strength and not to the presence of PpcC. For this reason, results for PpcC are only discussed when this protein is titrated and not the titrant.

Table 6.1 - Midpoint and individual heme reduction potential values of cytochromes PpcA-E. All values (*versus* standard hydrogen electrode) refer to pH 7. ND stands for not determined. In the periplasmic cytochromes, the hemes are numbered I, III and IV as consequence of superimposing the hemes with those of structurally homologous tetraheme cytochromes *c3* [55].

Cytochrome	Midpoint redox potential	Reduction potential values of the hemes [56]		
	E_{app}	I	III	IV
PpcA	-117	-147	-104	-110
PpcB	-137	-146	-155	-119
PpcC	-143	ND	ND	ND
PpcD	-132	-149	-96	-151
PpcE	-134	-154	-160	-96

The NMR chemical shift perturbation of the heme methyl signals was measured by comparing the resonances of the 1D ^1H -NMR spectrum of each isolated cytochrome with those obtained for a 1:3 ratio (at higher ratios the superimposition of signals allows monitoring less resonances) and are indicated in Figure 6.8A. Overall, the chemical shift perturbation values are within the range of reported transient protein interactions [57], since their heme methyl resonances undergo very small chemical shift variation throughout the experiments (see also Figures 8.2-8.6, Appendix). The exception is PpcC that shows larger perturbations (Figure 8.4, Appendix), but these are probably associated with additional variations in ionic strength as discussed above. For some of the titrations, there is a tendency to favor the interaction with one of the hemes, as depicted in Figure 6.8. This is the case of heme I for PpcB and PpcC, and heme IV for PpcE (Figure 6.8B). However, in all the titrations, signals from the three heme groups are slightly affected, pointing for the formation of nonspecific protein complexes.

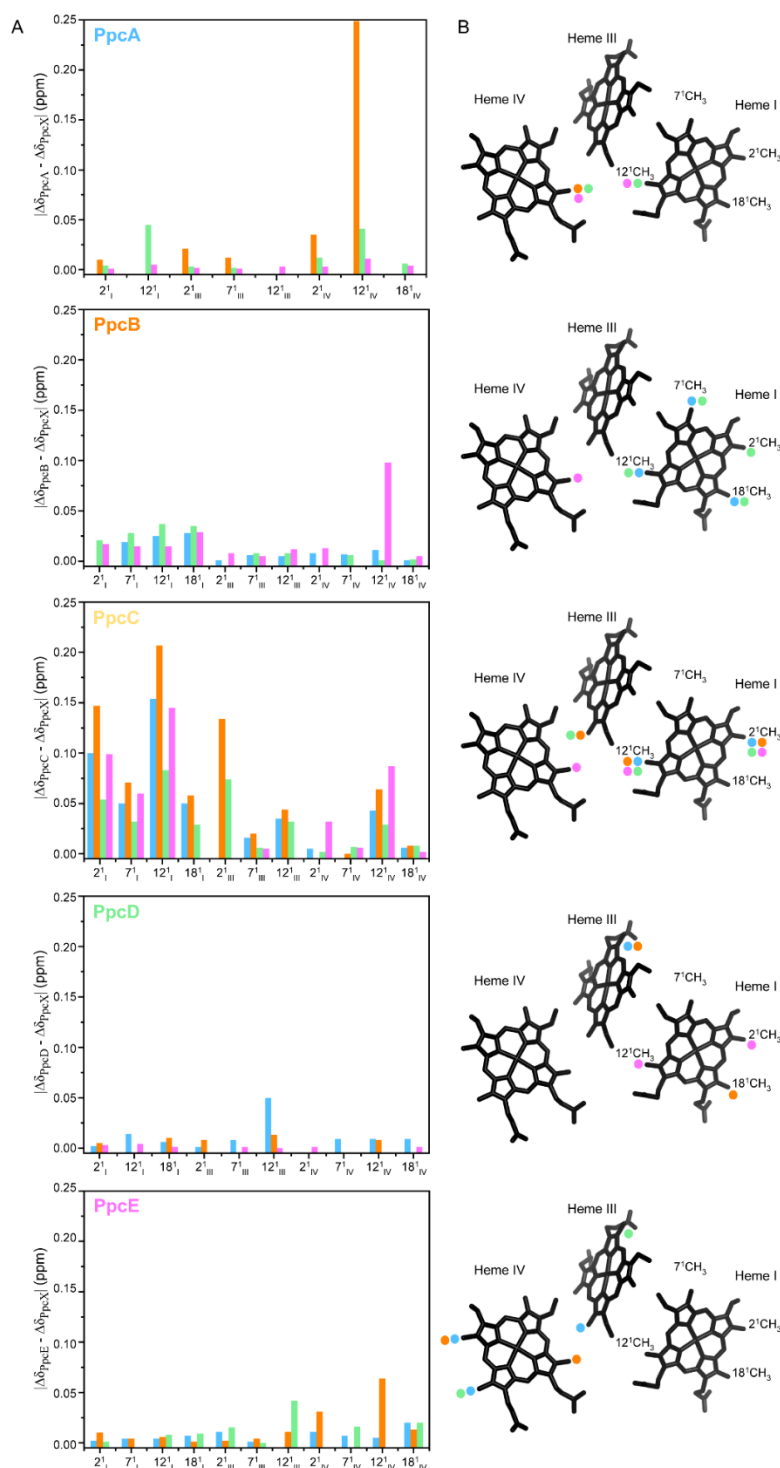


Figure 6.8 - Interacting interfaces of cytochromes PpcA-E. (A) The histograms compare the heme methyl's chemical shift variations in the oxidized state calculated from the data obtained at a 1:3 ratio. Each triheme cytochrome is labelled by a different color and the color code is respected in all figures: PpcA (blue), PpcB (orange), PpcC (yellow), PpcD (green) and PpcE (pink). The heme methyls are labeled according to the IUPAC-IUB nomenclature for tetrapyrroles [37]. (B) Representation of the most affected heme methyls residues in the heme core of each cytochrome. The most affected groups in each titration are indicated by a colored circle and were selected by calculating the cut-off value for each set of experiments following the procedure reported by Schumann and co-workers [58]. The cut-off values $\Delta\delta_{\text{cut-off}}$ (in ppm) are the following: (PpcA/PpcB) 0.113, (PpcA/PpcD) 0.024, (PpcA/PpcE) 0.005, (PpcB/PpcA) 0.014, (PpcB/PpcD) 0.021, (PpcB/PpcE) 0.034, (PpcC/PpcA) 0.068, (PpcC/PpcB) 0.099, (PpcC/PpcD) 0.042, (PpcC/PpcE) 0.073, (PpcD/PpcA) 0.018, (PpcD/PpcB) 0.009, (PpcD/PpcE) 0.002, (PpcE/PpcA) 0.009, (PpcE/PpcB) 0.023 and (PpcE/PpcD) 0.019. For a guide the heme methyls are indicated on heme I of each heme core.

Given the small chemical shift variations observed, and the transient nature of the redox complexes formed between each pair of triheme cytochromes, we complemented these experiments with electron transfer reactions experiments with NMR to confirm that in addition to the formation of the complex there are in fact electron transfer events. In these experiments the differences between the NMR fingerprints of each triheme cytochrome in the reduced and oxidized states are explored. This strategy has been applied to identify some physiological redox partners in *G. sulfurreducens*, as it was the case of PpcA/OmcF, CbcL/PpcA, ImcH/PpcA and PpcA-E/MacA [59-62]. In these conditions, the proteins forming the complex are studied in their physiological natural state, i.e., one reduced and the other oxidized, which effectively validates the indication given by the chemical shift perturbation experiments and eliminates the false positive results. Indeed, for a particular pair of cytochromes forming an effective transient complex, after the addition of an oxidized cytochrome to a reduced one, the typical signals of the reduced form of the first and those of the oxidized form of the second will be observed in the 1D ^1H -NMR spectrum. Thus, in the present work 20 independent assays were performed for the possible combinations of the five triheme cytochromes, by adding one oxidized cytochrome to a reduced one in anaerobic conditions (Figure 6.9 and Figures 8.8-8.11 of Appendix). All the cytochromes transferred electrons between each other, with both cytochromes attaining an intermediate state, and thus corroborating the interaction detected with both proteins in the oxidized state. The results are also in agreement with the comparable range of the reduction potential values covered by the heme groups and the overlapped redox windows of the different cytochromes (Table 6.1).

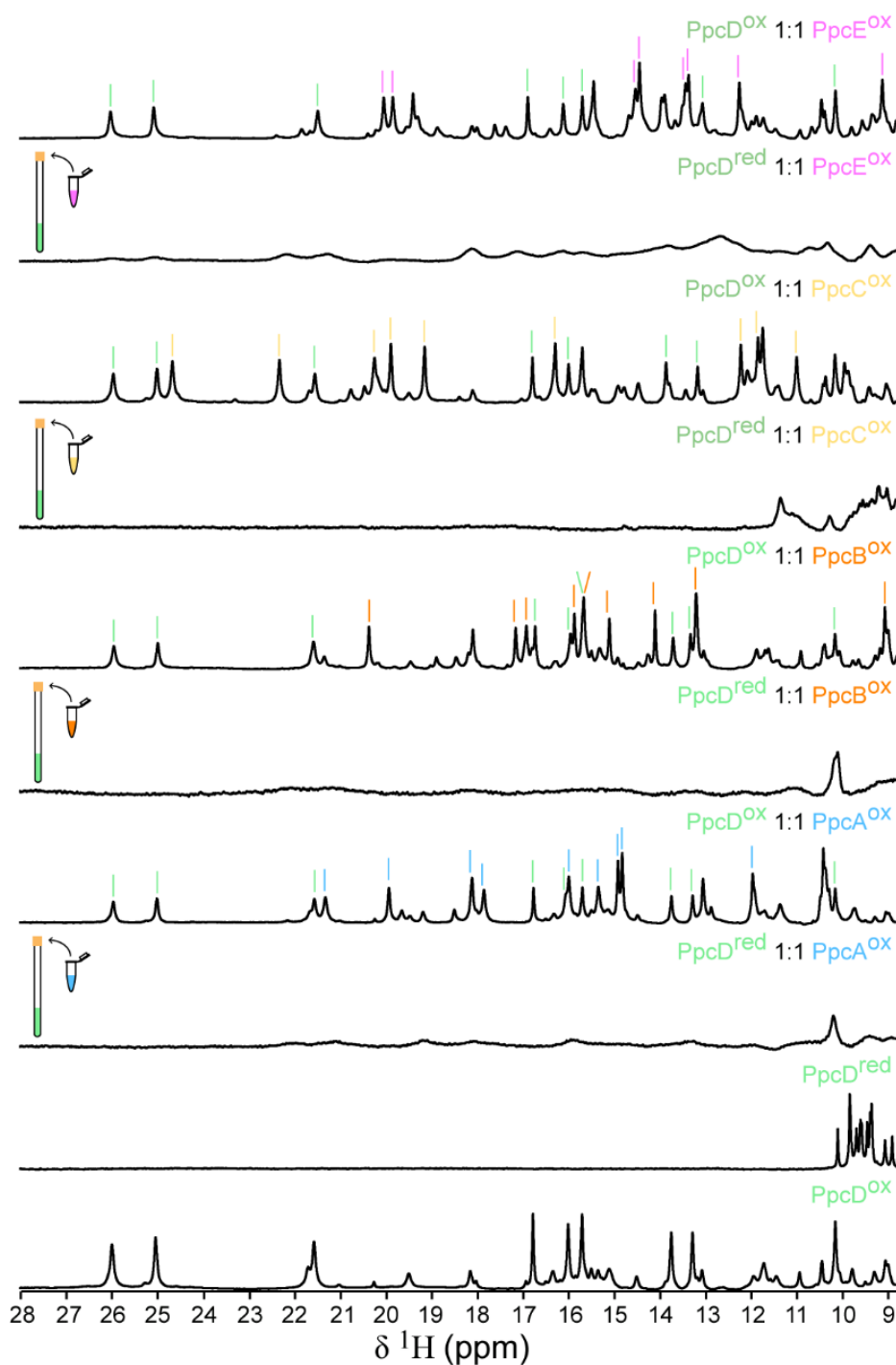


Figure 6.9 - Electron transfer reactions between cytochrome PpcD and the other triheme cytochrome family members monitored by NMR. The 1D ^1H -NMR spectra of PpcD in the oxidized (PpcD^{ox}) and reduced (PpcD^{red}) state are shown in the bottom. For each pair, spectra obtained after addition of an equimolar amount of the other cytochrome in the oxidized state (PpcD^{red}:PpcX^{ox}) and after exposure to atmospheric O₂ (PpcD^{ox}:PpcX^{ox}) are successively indicated. In the latter spectrum, the heme methyls of the cytochromes are colored for identification.

6.2.3 Implications

The five triheme periplasmic *c*-type cytochromes of the PpcA-family from *G. sulfurreducens* are constitutively expressed, though at different levels, in the presence of soluble and insoluble terminal electron acceptors [3, 5, 63-65]. These cytochromes share a high degree of homology and their structures are comparable and more conserved in the region of heme IV, typically surrounded by a high number of positively charged lysine residues [42]. While the slightly different amino acid composition at specific positions, spatial disposition and heme solvent exposure account for the differences in the reduction potential values of the cytochromes, their redox-active windows are considerably overlapped (Table 6.1 and Figure 6.10), which is explained by the same type of heme axial coordination (His-His), high level of amino acid sequence identity and structural homology. Nonetheless, the spatial localisation of particular residues in the less conserved regions may favour preferential interactions with redox partners and, hence, more efficient electron transfer events. Biomolecular interaction studies probed by NMR were previously accomplished between the most abundant member of the family (PpcA) and other cytochromes [8, 59, 66]. Overall, the data obtained showed that PpcA forms functional low-affinity complexes with distinct periplasmic redox partners, including the dodecaheme cytochrome GSU1996, the diheme cytochrome *c* peroxidase MacA and the periplasmic nine-heme domain of the inner membrane cytochrome CbcL. This functional promiscuity of PpcA is also extended to the transient redox complexes established with several *G. sulfurreducens*' terminal electrons acceptors, namely AQDS [10, 67], Fe(III) citrate [11] and Fe-NTA [68]. In all these studies, the interactions were electrostatically driven by the positively charged lysine residues located in the vicinity of heme IV. Thus, given the structural similarity amongst all members of the PpcA-family in this region, it is conceivable that any cytochrome can form similar complexes. Biomolecular interactions between other members of the PpcA-family and both the electron acceptor Fe(III) citrate and the peroxidase MacA have been also studied [11, 62]. In the first case, the other studied cytochromes (PpcB or PpcE) also interacted with Fe(III) citrate in the same specific and well-defined patch involving the positively charged lysine residues near heme IV. In the second case, each of the five members of the PpcA-family were able to establish a transient complex with an interacting interface involving, in larger extent, heme IV – except for PpcC in which the interface of heme I was preferred – and the region of MacA's high potential heme. This study also indicated that the magnitude of the chemical shift perturbation values obtained for PpcA-family cytochromes was much smaller compared to the one observed for MacA, indicating that the triheme cytochromes can adopt a higher degree of orientations. However, the transient nature of the complexes, the small size and high solvent exposure of the hemes, as well as the comparable redox-active potential windows of the triheme cytochromes still allow productive encounters for fast electron transfer events in the redox complexes. Overall, the data obtained from biomolecular interaction studies also suggest a high level of promiscuity

between the PpcA-family members and their redox partners, since any of them can establish similar electron transfer redox complexes. All cytochromes are rich in positively charged residues, which have been identified as key players in driving the formation of the redox complexes by electrostatics and promoting effective encounters for each pair of cytochromes, which would be not attainable if strong and specific interactions were necessary.

Recent *in vivo* experiments carried out by Choi and co-workers [52] provided further evidence for the high promiscuity of the PpcA-family members, despite their different biochemical properties and expression profiles. In fact, the authors showed that single deletions of the cytochromes did not affect the reduction of Fe(III) citrate or Fe(III) oxide reduction, suggesting that no single gene was essential for these conditions and hence any triheme cytochrome can support full reduction of Fe(III) compounds. This data, together with the biomolecular interactions probed by NMR between the PpcA-family members and with Fe(III) citrate or the cytochrome MacA reveal broad promiscuity in the periplasmic electron transfer network of *G. sulfurreducens*. There are reports in the literature suggesting that small periplasmic redox proteins carriers are promiscuous and facilitate electron transfer events between distinct inner membrane donors and outer membrane terminal acceptors [8, 30, 69-72], an event that we designate as inter-promiscuity.

Our results showed that any pair of members from the PpcA-family cytochromes can interact with each other to form a periplasmic electron transfer network, rather than a specific chain with well-defined interactions between redox complex pairs. The interacting regions in the transient redox complexes are not highly specific and no-well defined orientations are required, which permits a high turnover and rapid exchange of electrons. Thus, in addition to the inter-promiscuity revealed by the interaction with multiple periplasmic partners, our results identify another level of promiscuity – intra-promiscuity (*i.e.*, between the members of the same family) – that provide an answer to the question why *Geobacter* cells produce such an array of periplasmic cytochromes at the same time.

The simultaneous presence of the five cytochromes may indicate that multiple routes for electron transfer are active in the periplasm, which can negatively affect the performance of the microorganism in the context of a particular biotechnological application [51]. However, our findings show that the apparent redundancy is in fact considerably advantageous for the cells, since the simultaneous presence of all cytochromes permits that any yet unidentifiable metabolic route(s) can be promptly triggered, followed by the concomitant readjust of the expression levels of a particular cytochrome and thus conferring advantages to the cells [73]. In addition, the five cytochromes can establish a periplasmic electron transfer network covering a larger range of redox potential values than if it was formed by a single cytochrome (Figure 6.10). The formation of this interacting network is possible due to their structural similarity of the cytochromes and to the comparable redox-active functional regions. Even more importantly, our results show that the cytochromes not only exchange electrons between each other, but also, they remain in intermediate levels of oxidations. This is crucial because

all cytochromes are not fully oxidized or fully reduced, and thus are functionally active (*i.e.*, capable of receiving and donating electrons). The comparable extent of electron exchange between each pair of cytochromes, the transient nature and lack of specificity of the complexes suggests that this network of interactions has not been optimized by evolution. A possible explanation for this is provided by the comparable structural and redox properties of the cytochromes that allow them to be compatible and set the periplasmic redox potential to an optimal value that the inner and outer membrane cytochromes are also fully functional, thus assuring a smooth electron transfer from the interior to the cell exterior (see Figure 6.10).

The small size of the cytochromes and the high solvent exposure of the redox centers eliminate the number of nonfunctional events and overcome the apparent need for strictly well-defined and linear pathways in the periplasmic space as universally thought. Therefore, evolution regarding the expression of a unique member of the family by the cells was not necessary to optimize since having all members present is indeed advantageous. The results show that the build-up of the periplasmic network is non-specific and can be established by the complementary transient interaction between any pair of periplasmic cytochromes and it is not dependent of the molecular tumbling (*i.e.* diffusion) of each protein. The periplasmic pool of triheme cytochromes supplies the necessary reducing power to *G. sulfurreducens* cells guaranteeing that electrons will cross the periplasm, regardless of the terminal acceptor.

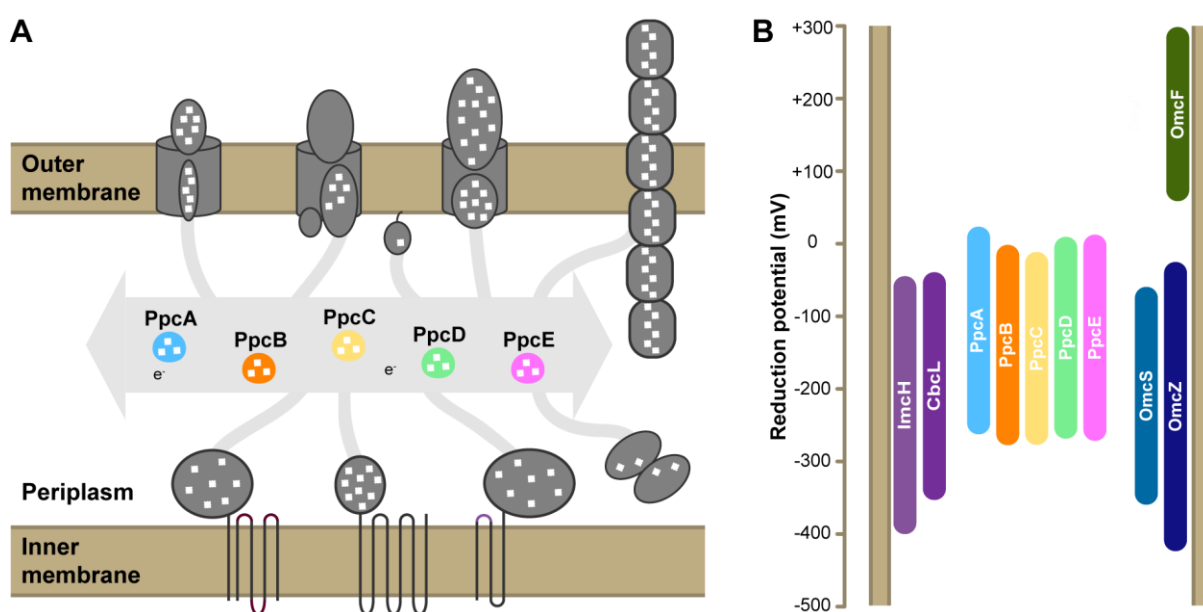


Figure 6.10 - Intra-promiscuity in the periplasmic space of *G. sulfurreducens*. (A) Different inner membrane and outer membrane associated cytochromes were shown to be involved in specific electron transfer pathways; however, promiscuity was observed with the periplasmic family of triheme cytochromes (inter-promiscuity). The interactions observed in the current study reveal that cytochromes from the same family interact (intra-promiscuity) and contribute to the maintenance of a functional redox potential in the cell. *c*-type heme groups are represented in white. (B) Redox-active windows of inner membrane cytochromes CbcL and ImcH [59, 61], periplasmic PpcA-family [27], nanowire cytochromes OmcS and OmcZ [74, 75] and outer membrane associated OmcF [27].

6.3 References

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CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 Conclusions and future perspectives

In the last two decades our group has focused on the study of electron transfer proteins from the bacterium *G. sulfurreducens*. This long journey started with the characterization of the whole family of periplasmic triheme cytochromes (PpcA-E). Then we move to the characterization of other proteins that also participate in the EET pathways, such as the monoheme cytochrome OmcF and the dodecaheme cytochrome GSU1996. Functional and structural characterization of these proteins was obtained and NMR-based methods for isotopic labelling, straightforward assignment of the heme signals in the oxidized state, and more recently, a methodology to directly probe electron transfer reaction between putative redox partners by NMR were developed. At the start of this PhD Thesis, biomolecular interactions studies started to emerge in the group. This was a natural follow up of our research since after determining the functional properties of a large group of proteins, their structures and NMR fingerprints, the logical step will be the probe between electron transfer proteins that share the same electron transfer pathway, including terminal acceptors.

From this point it was also an objective to move from *G. sulfurreducens* to other *Geobacter* strains to evaluate the similarity, if any, between their respiratory process. *G. metallireducens* was my target because it can oxidize a larger number of organic compounds compared to *G. sulfurreducens* species, including aromatic and non-aromatic compounds. In addition, evidence indicated that *G. metallireducens* is more versatile in DIET events, for example with *Methanosaeta* species in the conversion of wastewaters' organic compounds to methane, an important process used for bioenergy production. Therefore, this larger respiratory versatility prompted us to initiate the study of electron transfer mechanisms in *G. metallireducens*.

We started with the same approach previously used for *G. sulfurreducens*, *i.e.*, by the study of the most abundant periplasmic triheme cytochromes, except for PpcA which have been already characterized in the group. For the other four members of the family different levels of achievement were attained. For PpcB, PpcC e PpcE it was possible to improve their expression yields, to determine the type of axial coordination and spin state of the hemes. In the case of PpcC and PpcE the heme substituent NMR signals were also assigned. Within this set of cytochromes, PpcE reached the more advanced state of characterization, which includes the determination of its reduction potential values at pH 7 and 8 and assignment of the heme substituents in the reduced form. A very unusual behavior prevents its further characterizations, namely at the level of the determination of the redox potential of the heme groups because the heme signals of at least one heme become extremely broad to be detected. In this point, it will be interesting in the future to understand the rational of this unprecedented behavior and establish the necessary experimental conditions to permit such characterization. Even though, the difference between the redox potential values at pH 7 and 8 for PpcE was never observed for a triheme cytochrome, included those from *G. sulfurreducens*, indicating that PpcE from *G. metallireducens* has a particular functional

modulation of its properties with the pH that require further study. This will include the identification of the redox-Bohr center(s) and eventually the determination of its structure at different pH values.

Within the scope of this Thesis the most complete characterization of a member of the PpcA-family from *G. metallireducens* was obtained for PpcF. It was possible to determine the redox potential values of each heme group, as well as their redox and redox-Bohr interactions. At structural level we were able to determine the orientation of the heme axial histidine planes and the magnetic properties of the cytochrome. In addition, with the analysis of the protein NMR signals (hemes, backbone and side chains) it was possible to conclude that the overall structure of PpcF is conserved compared to the cytochromes of the PpcA-family from *G. sulfurreducens*, a feature previously observed for cytochrome PpcA from the *G. metallireducens*. This was also confirmed by AlphaFold models for the other members of the family.

Interestingly, PpcA and PpcF from *G. metallireducens* showed redox potentials much less negative, compared to the PpcA-family of *G. sulfurreducens*, which hint, at least, a different redox working potential range of the bacteria. In the future, a natural line of research will include the characterization of inner and outer membrane associated cytochromes to evaluate if functional diversity is also present at these levels. Then, a fully structural and functional characterization of all members of the PpcA-family from *G. metallireducens* will be important but the data already obtained for PpcA and PpcF can be used to elucidate the electron transfer pathways in *G. metallireducens*. This will include the monitorization of biomolecular interactions between PpcA-family members and membrane associated cytochromes and eventually with other periplasmic cytochromes. Having been established that pili protein is part of the secretory system of electrically conductive filaments made of *c*-type cytochromes polymers, interactions between periplasmic cytochromes and OmcE/OmcZ filaments, already identified in *G. metallireducens*, must be pursuit. Regarding the PilA protein it will be interesting to study the interactions between the heterodimer PilA-N/C and filaments OmcE/Z.

Finally, the work developed in this Thesis also contributed to the elucidation of electron transfer pathways in *G. sulfurreducens* by answering one of the questions that has always been intriguing to the scientific community “Why *Geobacter* express highly structurally homologous cytochromes constitutively in the periplasm?” and this apparent redundancy is indeed advantageous for the cells, as it allows any metabolic pathway to be triggered. Also, the five cytochromes cover a wider range of redox potential values than if there were only a single cytochrome. Furthermore, it was shown that the abundant cytochrome PpcA can function as a terminal reductase. In fact, by coupling UV-visible studies and NMR biomolecular interaction experiments between this cytochrome and the electron acceptor Fe-NTA it was discovered that this molecule can be directly reduced by the cytochrome and to identify the heme and the regions that are responsible for the formation of this redox complex.

| 8.

APPENDIX

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8.1 Supplementary figures

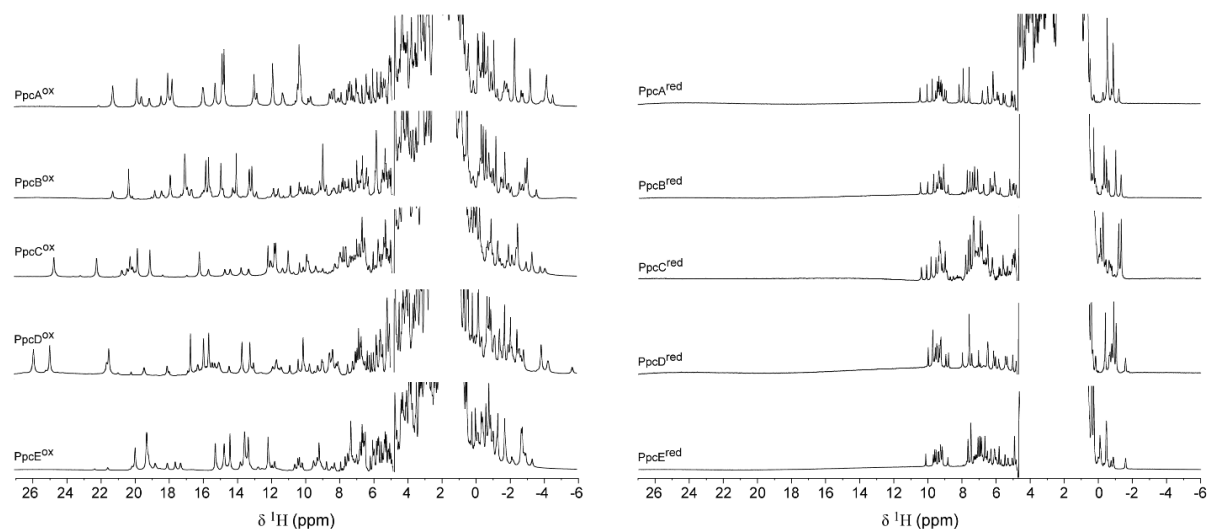


Figure 8.1 - 1D ^1H -NMR spectra of cytochromes PpcA-E from *G. sulfurreducens* in the oxidized (left) and reduced (right) states. All spectra were acquired at 25 °C in 8 mM sodium phosphate buffer pH 7.4 with NaCl (final ionic strength 20 mM).

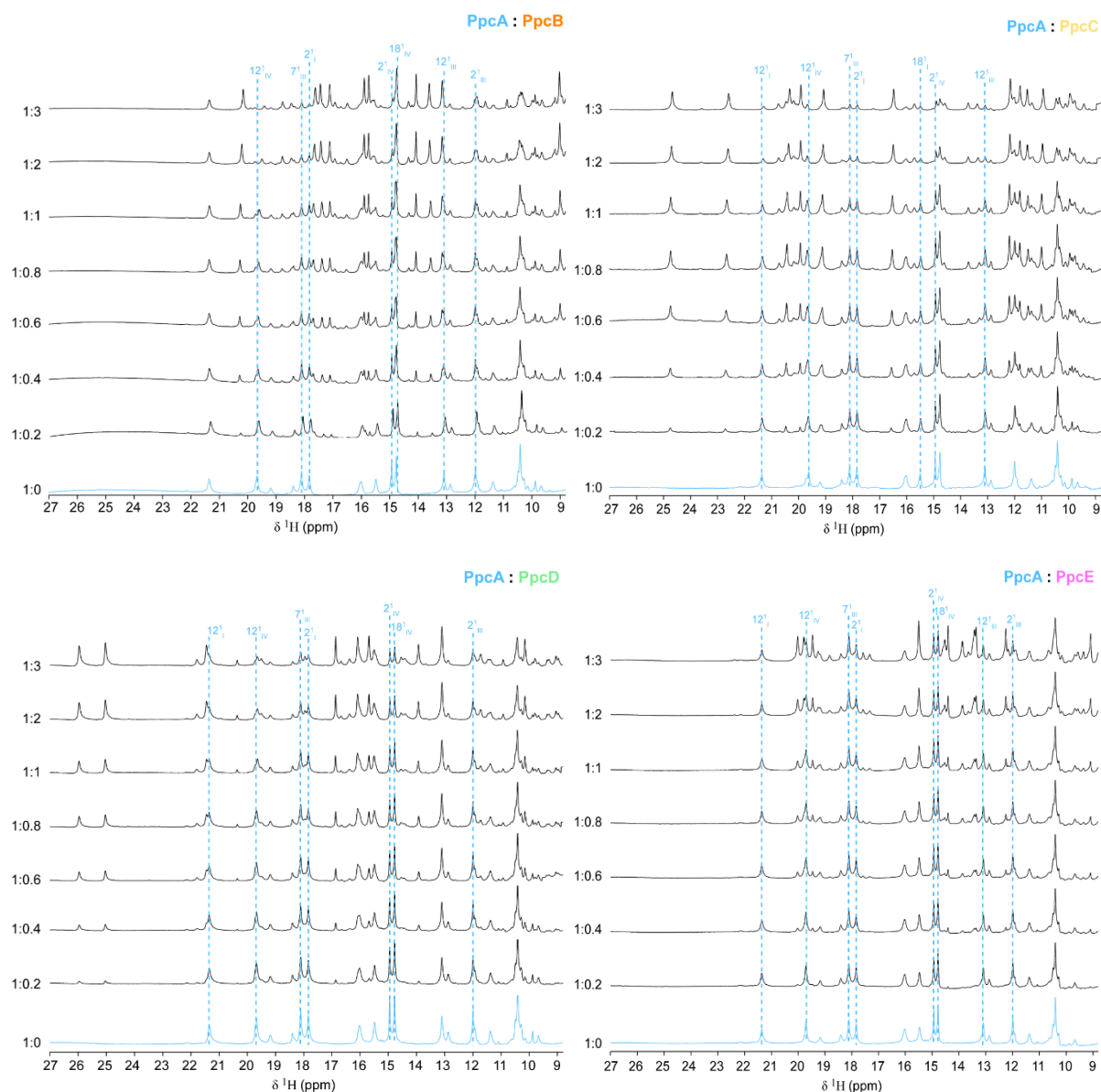


Figure 8.2 - Expansions of the low-field regions of the 1D ^1H -NMR spectra of PpcA obtained for the chemical shift perturbation studies with the other triheme cytochrome family members. The 1D ^1H -NMR spectra of PpcA in the presence of increasing amounts of each of the other cytochrome are shown. The molar ratios between the pairs of cytochromes are indicated on the left side of each spectrum. The heme methyls of the titrated cytochromes are labeled.

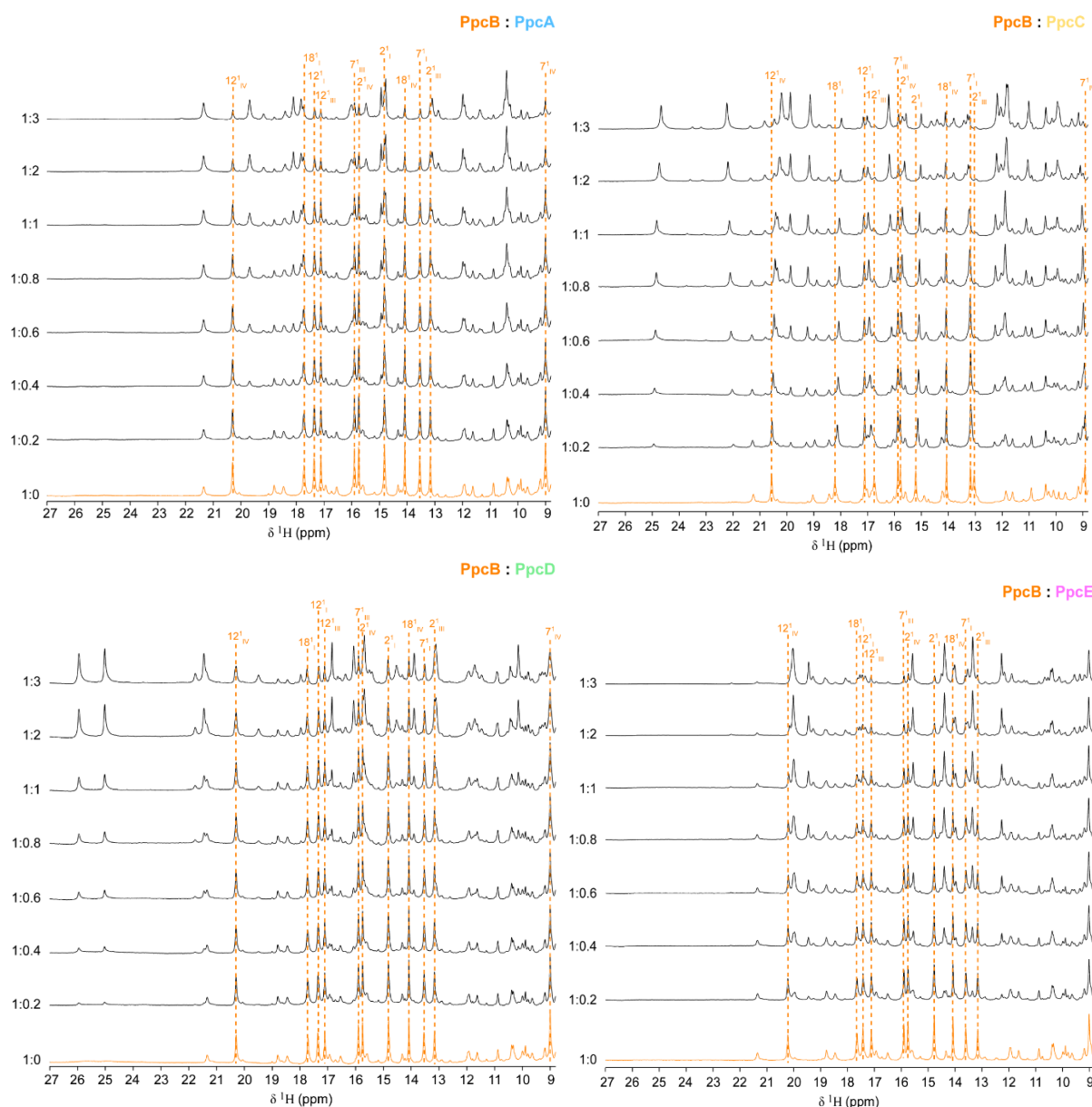


Figure 8.3 - Expansions of the low-field regions of the 1D ^1H -NMR spectra of PpcB obtained for the chemical shift perturbation studies with the other triheme cytochrome family members. The 1D ^1H -NMR spectra of PpcB in the presence of increasing amounts of each of the other cytochrome are shown. The molar ratios between the pairs of cytochromes are indicated on the left side of each spectrum. The heme methyls of the titrated cytochromes are labeled.

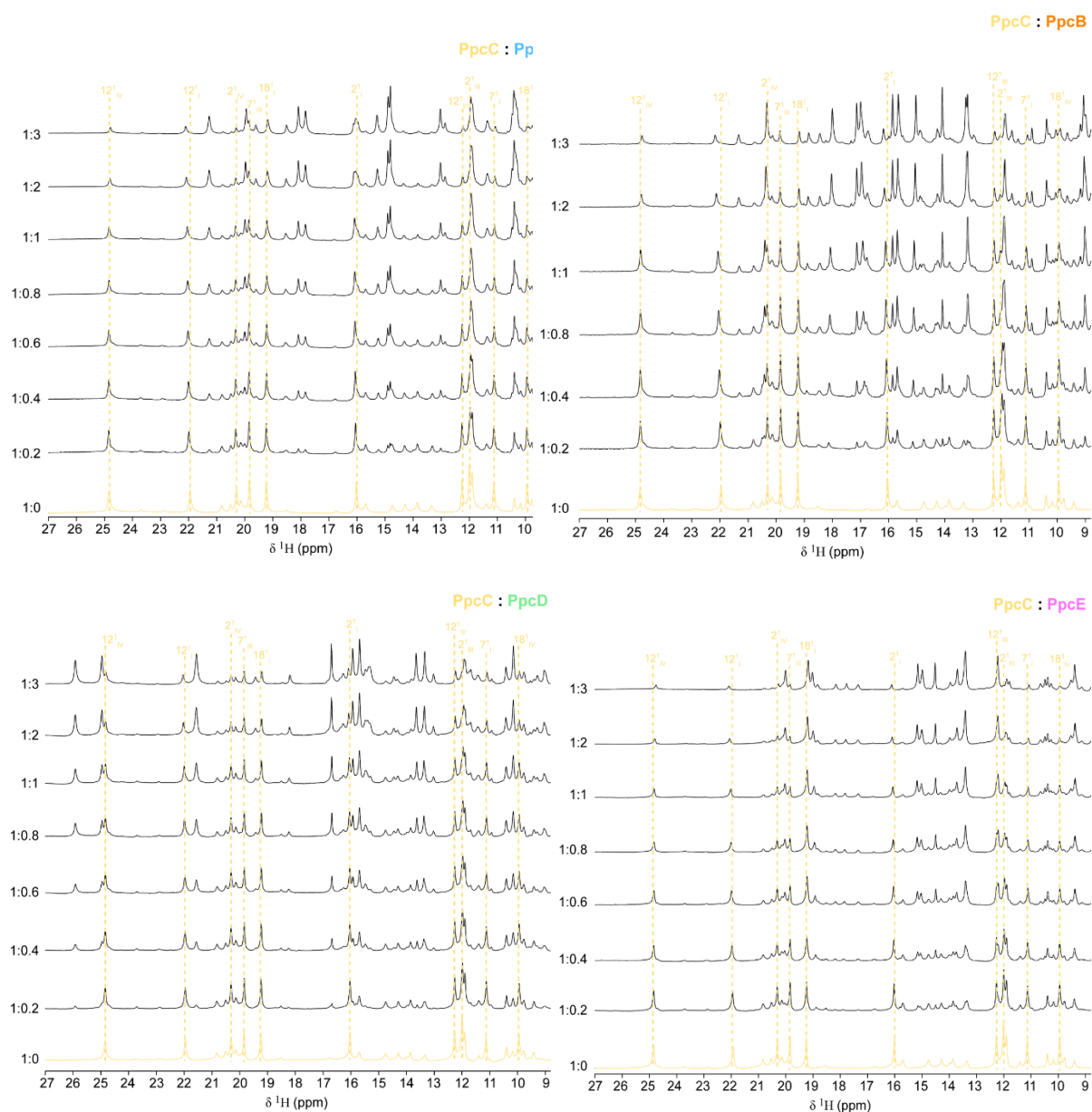


Figure 8.4 - Expansions of the low-field regions of the 1D ^1H -NMR spectra of PpcC obtained for the chemical shift perturbation studies with the other triheme cytochrome family members. The 1D ^1H -NMR spectra of PpcC in the presence of increasing amounts of each of the other cytochrome are shown. The molar ratios between the pairs of cytochromes are indicated on the left side of each spectrum. The heme methyls of the titrated cytochromes are labeled.

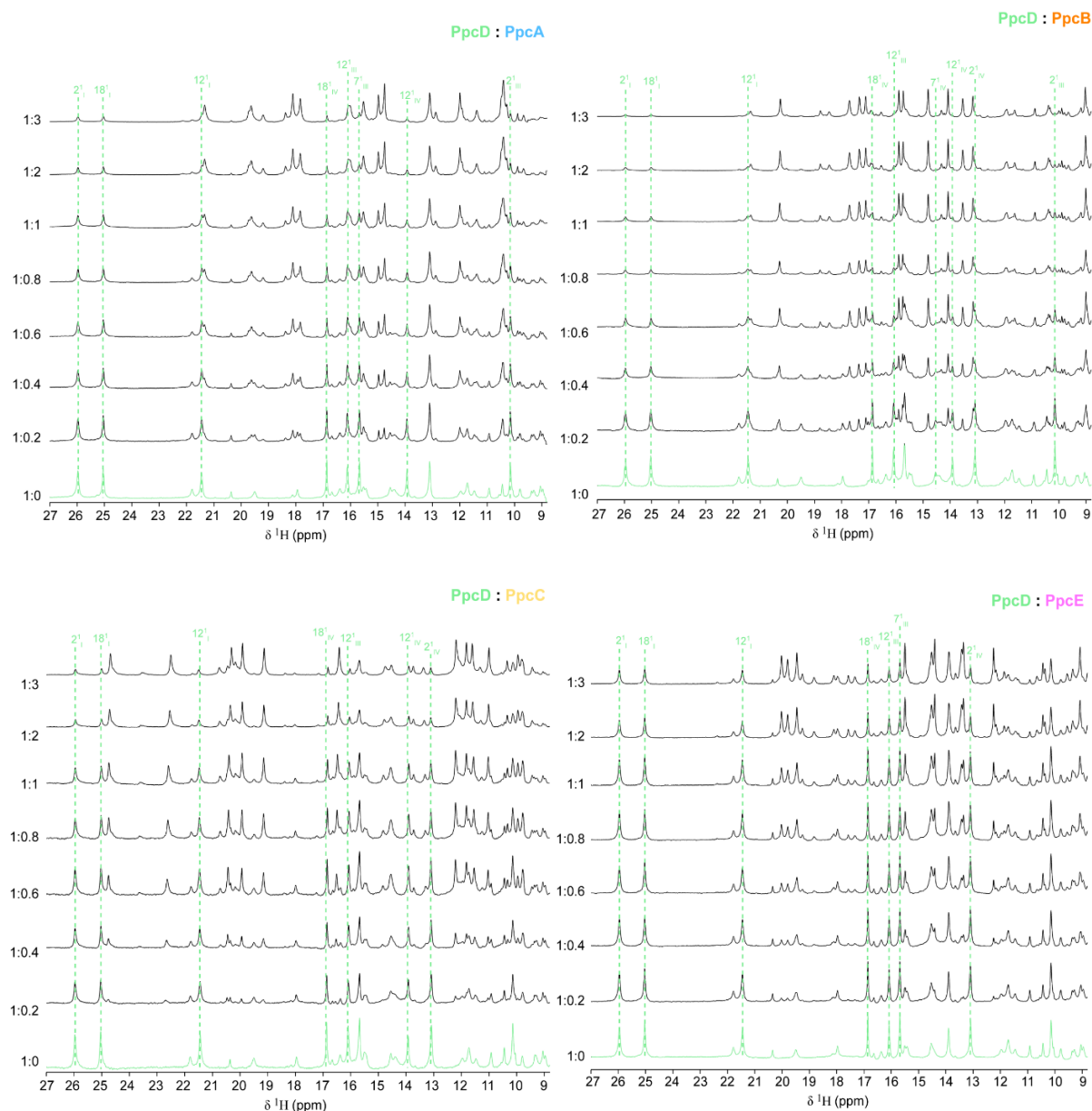


Figure 8.5 - Expansions of the low-field regions of the 1D ^1H -NMR spectra of PpcD obtained for the chemical shift perturbation studies with the other triheme cytochrome family members. The 1D ^1H -NMR spectra of PpcD in the presence of increasing amounts of each of the other cytochrome are shown. The molar ratios between the pairs of cytochromes are indicated on the left side of each spectrum. The heme methyls of the titrated cytochromes are labeled.

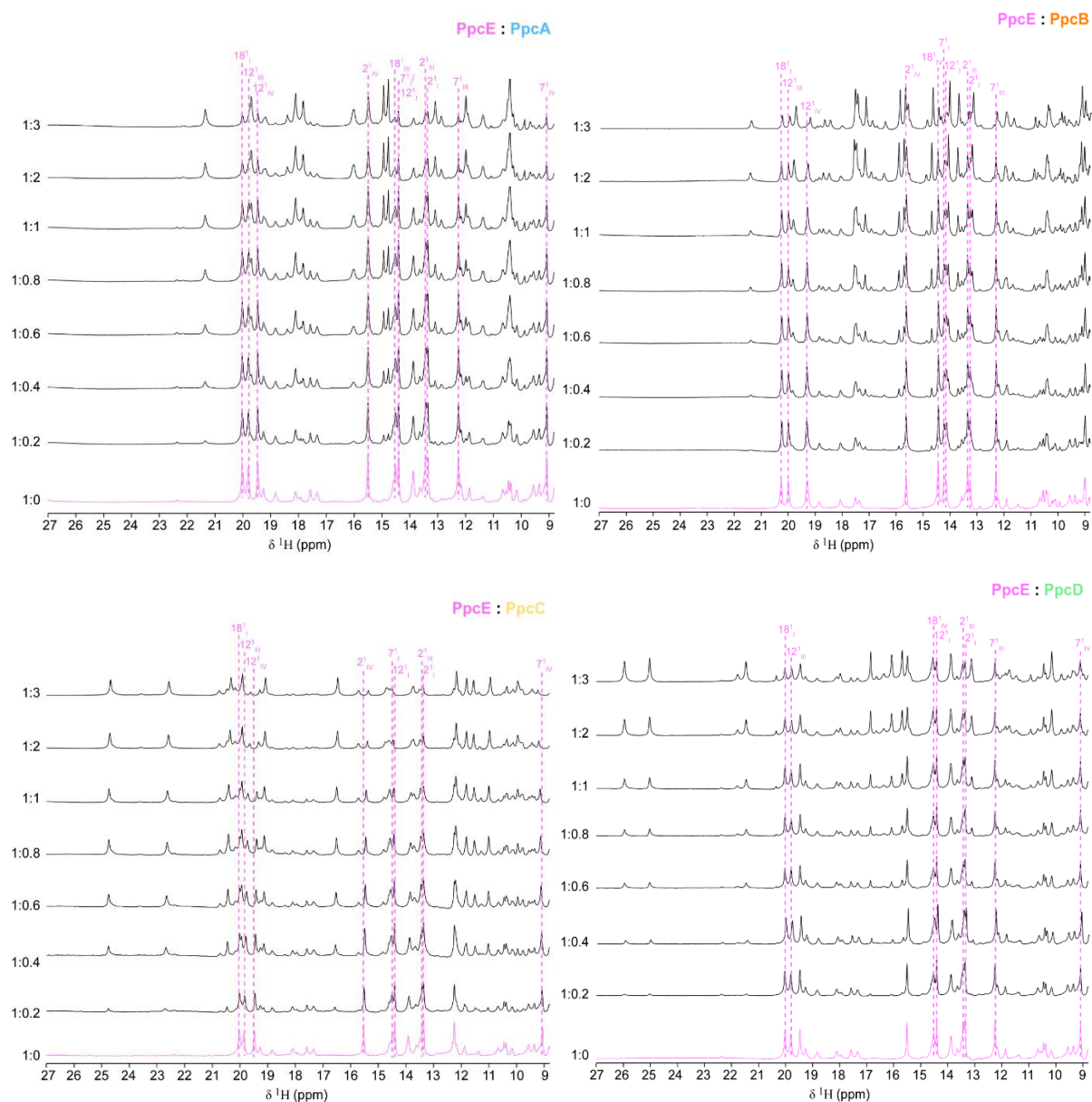


Figure 8.6 - Expansions of the low-field regions of the 1D ^1H -NMR spectra of PpcE obtained for the chemical shift perturbation studies with the other triheme cytochrome family members. The 1D ^1H -NMR spectra of PpcE in the presence of increasing amounts of each of the other cytochrome are shown. The molar ratios between the pairs of cytochromes are indicated on the left side of each spectrum. The heme methyls of the titrated cytochromes are labeled.

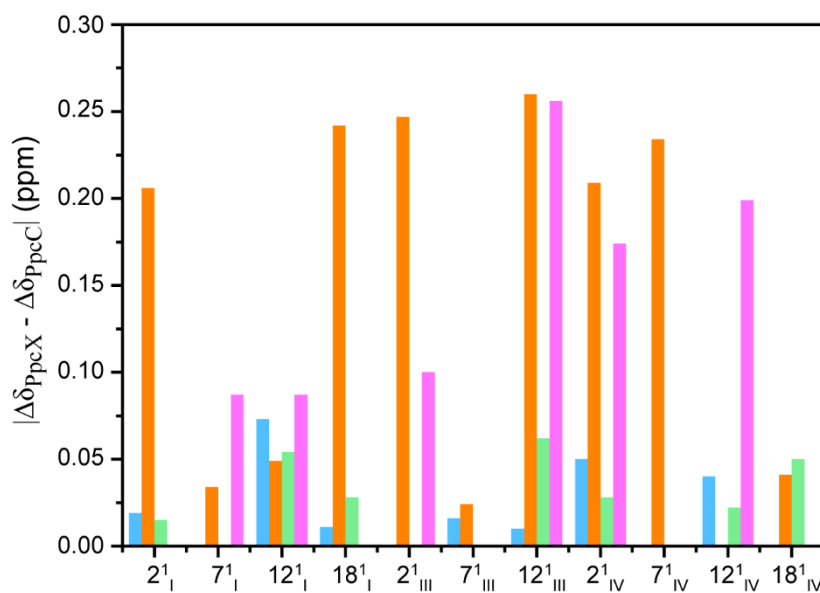


Figure 8.7 - NMR Chemical shift perturbation studies obtained with PpcC as the titrant. The histograms compare the heme methyl's chemical shift variations in the oxidized state calculated from the data obtained at 1:3 ratio. Each triheme cytochrome is labelled by different colors: PpcA (blue), PpcB (orange), PpcD (green) and PpcE (pink).

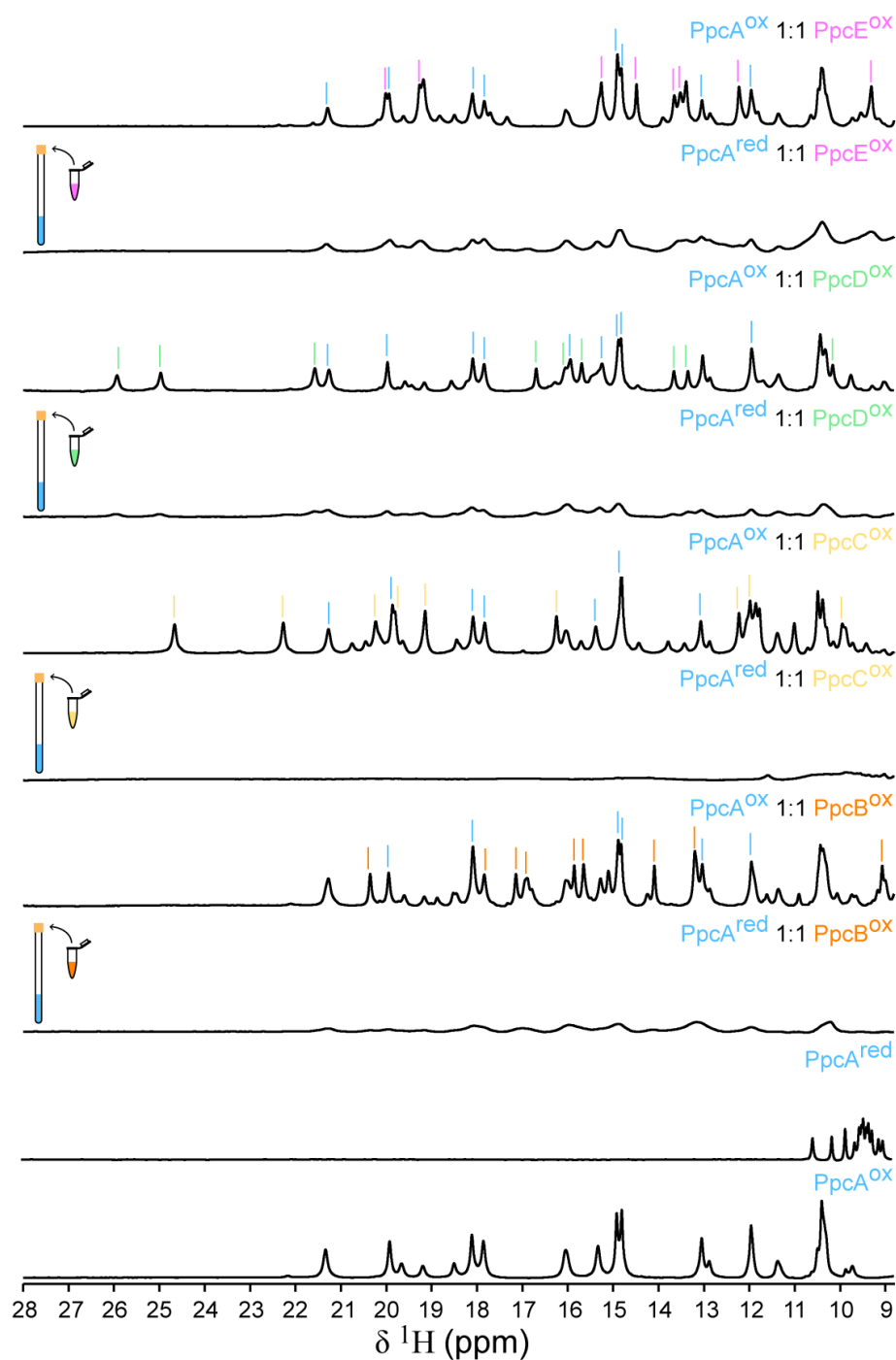


Figure 8.8 - Electron transfer reactions between cytochrome PpcA and the other triheme cytochrome family members monitored by NMR. The 1D ^1H -NMR spectra of PpcA in the oxidized (PpcA^{ox}) and reduced (PpcA^{red}) state are shown in the bottom. For each pair, spectra obtained after addition of an equimolar amount of the other cytochrome in the oxidized state ($\text{PpcA}^{\text{red}}:\text{PpcX}^{\text{ox}}$) and after exposure to atmospheric O_2 ($\text{PpcA}^{\text{ox}}:\text{PpcX}^{\text{ox}}$) are successively indicated. In the latter spectrum, the heme methyls of the cytochromes are colored for identification.

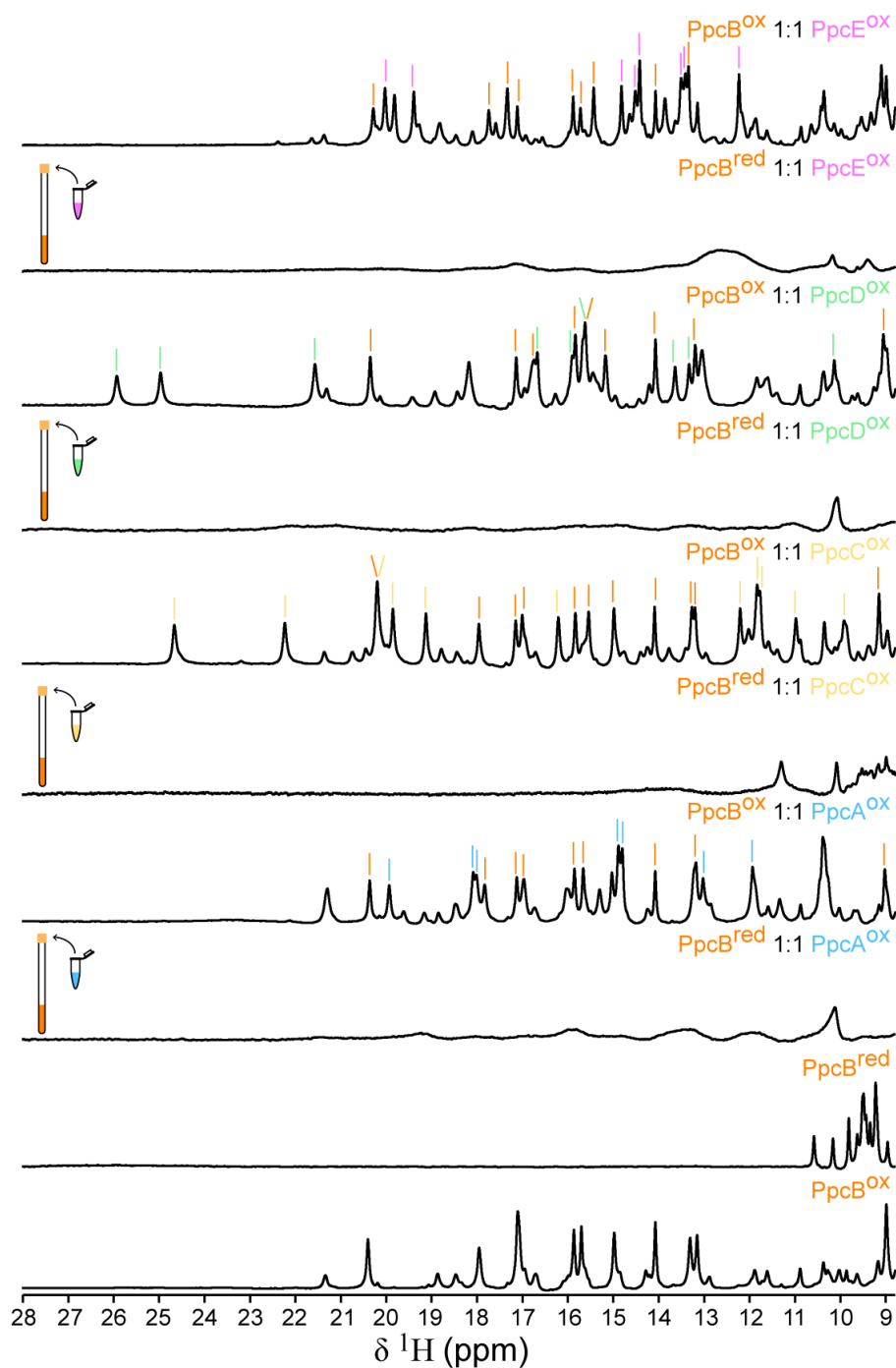


Figure 8.9 - Electron transfer reactions between cytochrome PpcB and the other triheme cytochrome family members monitored by NMR. The 1D ^1H -NMR spectra of PpcB in the oxidized (PpcB^{ox}) and reduced (PpcB^{red}) state are shown in the bottom. For each pair, spectra obtained after addition of an equimolar amount of the other cytochrome in the oxidized state ($\text{PpcB}^{\text{red}}:\text{PpcX}^{\text{ox}}$) and after exposure to atmospheric O₂ ($\text{PpcB}^{\text{ox}}:\text{PpcX}^{\text{ox}}$) are successively indicated. In the latter spectrum, the heme methyls of the cytochromes are colored for identification.

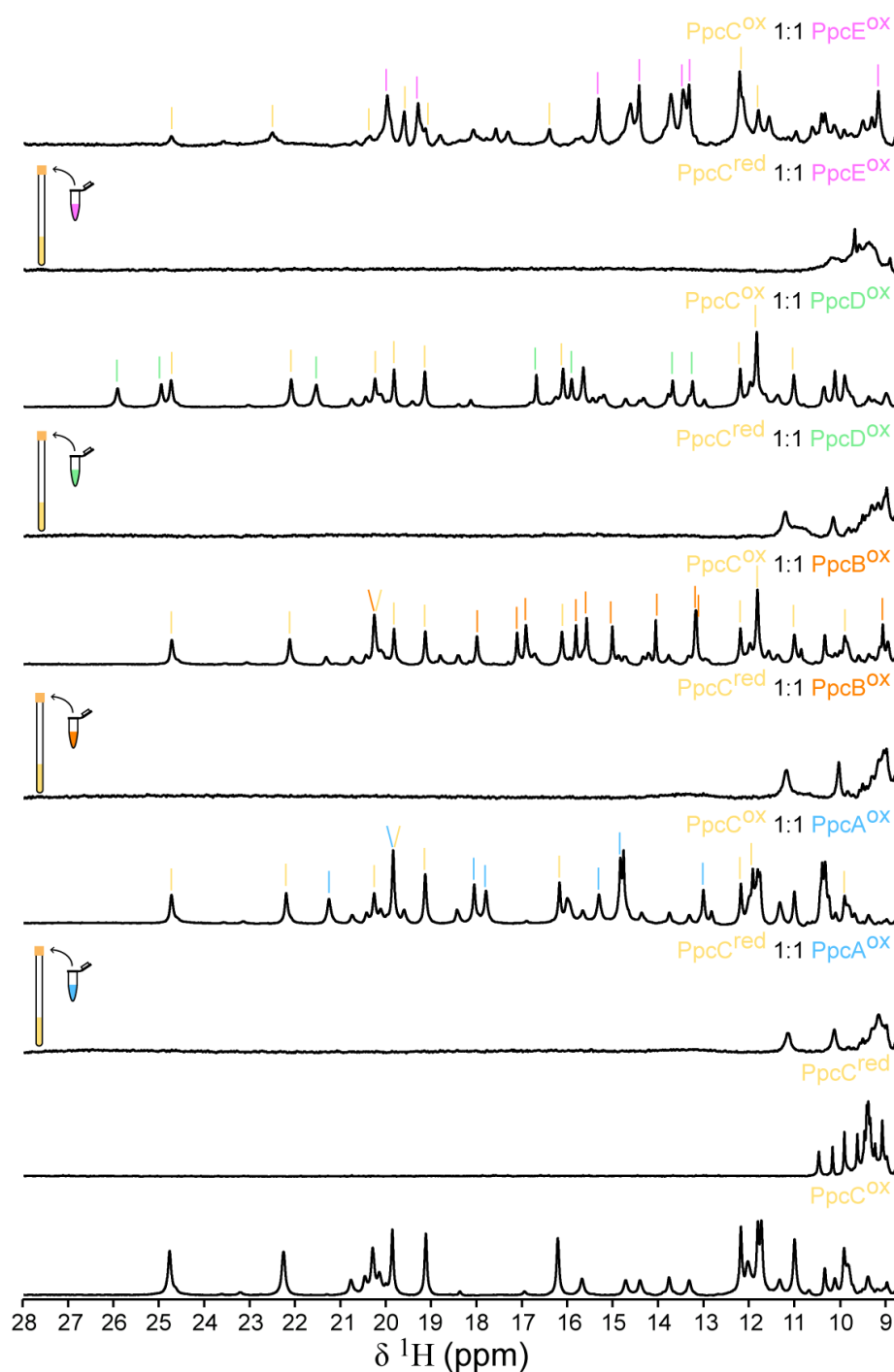


Figure 8.10 - Electron transfer reactions between cytochrome PpcC and the other triheme cytochrome family members monitored by NMR. The 1D ^1H -NMR spectra of PpcC in the oxidized (PpcC^{ox}) and reduced (PpcC^{red}) state are shown in the bottom. For each pair, spectra obtained after addition of an equimolar amount of the other cytochrome in the oxidized state ($\text{PpcC}^{\text{red}}:\text{PpcX}^{\text{ox}}$) and after exposure to atmospheric O_2 ($\text{PpcC}^{\text{ox}}:\text{PpcX}^{\text{ox}}$) are successively indicated. In the latter spectrum, the heme methyls of the cytochromes are colored for identification.

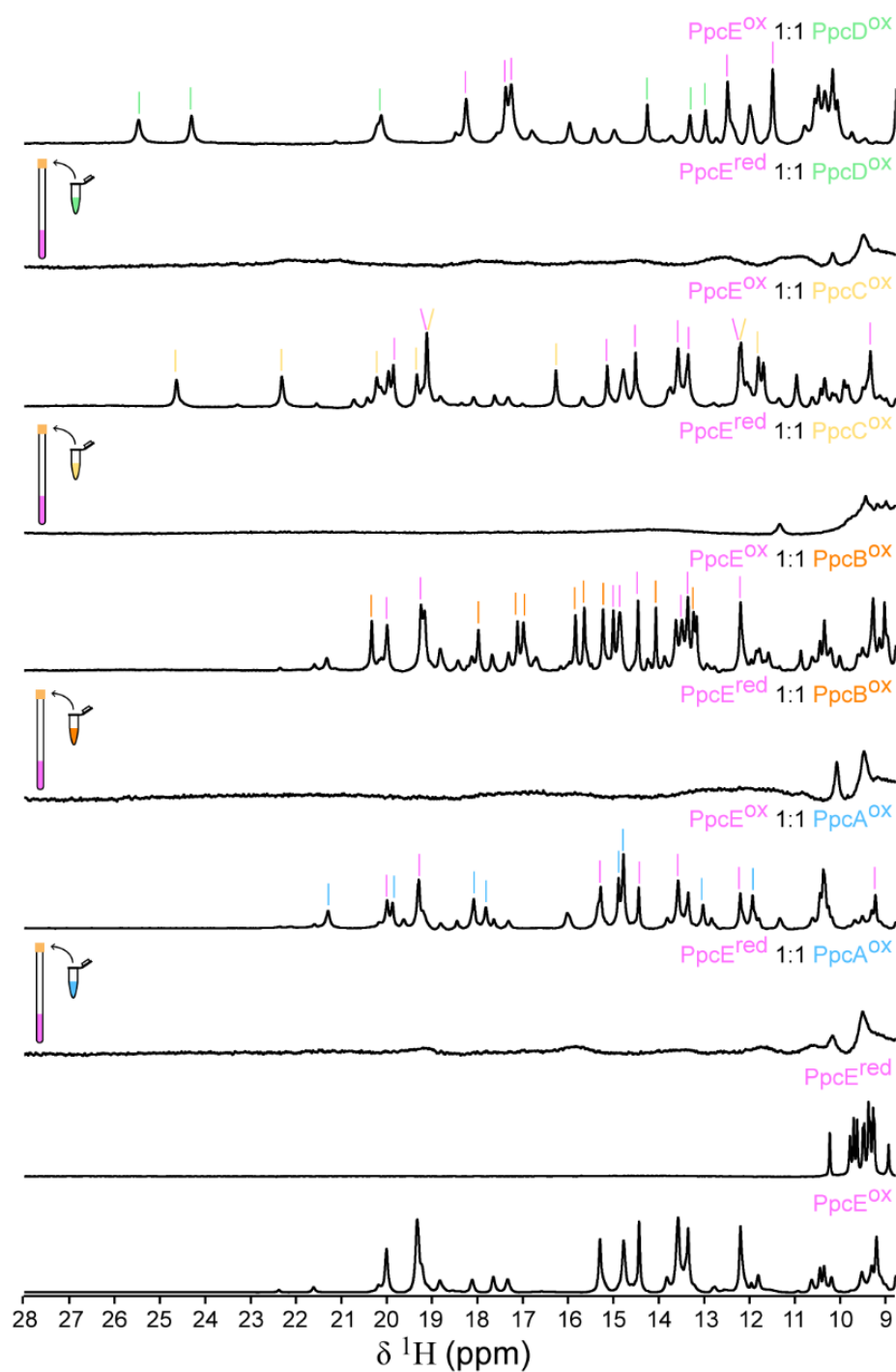


Figure 8.11 - Electron transfer reactions between cytochrome PpcE and the other triheme cytochrome family members monitored by NMR. The 1D ^1H -NMR spectra of PpcE in the oxidized (PpcE^{ox}) and reduced (PpcE^{red}) state are shown in the bottom. For each pair, spectra obtained after addition of an equimolar amount of the other cytochrome in the oxidized state ($\text{PpcE}^{\text{red}}:\text{PpcX}^{\text{ox}}$) and after exposure to atmospheric O_2 ($\text{PpcE}^{\text{ox}}:\text{PpcX}^{\text{ox}}$) are successively indicated. In the latter spectrum, the heme methyls of the cytochromes are colored for identification.

8.2 Supplementary tables

Table 8.1 – Backbone and side chain assignment of reduced cytochrome PpcF from *G. metallireducens* (45 mM sodium phosphate buffer pH 7.1, 100 mM ionic strength at 25 °C).

Residue number	Residue type	Atom	Chemical shift (ppm)	Residue number	Residue type	Atom	Chemical shift (ppm)
2	ASP	CA	55.81	6	PHE	HB2	3.38
		CB	40.31			HB3	4.37
		HA	3.66			HD2	7.85
		HB2	2.09			HE2	7.45
		HB3	2.20			HZ	7.63
3	VAL	CA	60.59			HN	9.66
		CB	35.07			NH	125.81
		CG1	21.01	7	PRO	CA	62.54
		CG2	21.52			CB	31.22
		HA	3.76			CD	51.20
		HB	1.53			CG	27.33
		HN	7.22			HA	4.52
		NH	119.24			HB2	2.17
		QG1	0.47			HD2	4.21
		QG2	0.52			HD3	4.30
4	PHE	CA	54.33			HG2	2.22
		CB	42.20			HG3	2.32
		HD2	5.52	8	ALA	CA	51.58
		HE2	6.07			CB	20.64
		HZ	6.68			HA	4.65
		HN	8.10			HN	7.81
		NH	123.30			NH	121.20
5	GLU	CA	54.40			QB	0.81
		CB	31.83	9	SER	CA	61.17
		CG	36.71			CB	61.87
		HA	5.11			HA	4.07
		HB2	1.66			HB2	3.95
		HB3	1.90			HN	10.84
		HG2	2.00			NH	124.11
		HG3	2.11	10	MET	CA	53.47
		HN	8.57			CB	31.56
		NH	120.32			HA	3.85
						HB2	-1.07
6	PHE	CA	53.87			HB3	0.03
		CB	39.96			HG2	0.97
		HA	5.71				

Residue number	Residue type	Atom	Chemical shift (ppm)
10	MET	HG3	1.14
		HN	9.31
		NH	117.90
11	GLY	CA	43.22
		HA2	3.19
		HA3	3.22
		HN	6.72
		NH	106.31
12	LYS	CA	57.41
		CB	32.87
		CD	29.38
		CE	41.80
		CG	23.97
		HA	4.22
		HB2	1.66
		HD2	1.55
		HE2	2.80
		HE3	2.87
		HG2	1.22
		HN	7.80
		NH	127.13
13	VAL	CA	60.63
		CB	35.07
		CG1	20.99
		CG2	19.12
		HA	4.49
		HB	2.51
		HN	8.68
		NH	126.48
		QG1	1.73
		QG2	-0.68
		HN	8.41
		NH	118.71
14	THR	CA	63.43
		CB	68.48
		CG2	21.61
		HA	4.79
		HB	4.21
		QG2	1.10
14	THR	HN	9.38
		NH	126.72
15	PHE	CA	51.66
		CB	43.30
		HA	5.94
		HB2	3.07
		HB3	3.87
		HD1	6.65
		HD2	7.01
		HE1	6.34
		HE2	6.38
		HZ	2.72
16	PRO	HN	9.50
		NH	127.08
		CA	61.20
		CB	28.95
		CD	51.27
		CG	27.00
		HA	4.07
		HB2	1.71
		HG2	0.86
		HD2	3.06
17	HIS	CA	57.56
		CB	27.89
		HA	1.18
		HB2	0.30
		HB3	0.65
		HD1	8.54
		HD2	1.66
		HE1	1.75
18	LYS	HN	6.03
		NH	132.17
		CA	58.15
		CB	31.02
		CD	28.58
		CE	41.78
		CG	23.85
		HA	2.92
		HB2	0.92

Residue number	Residue type	Atom	Chemical shift (ppm)
18	LYS	HB3	1.14
		HD2	1.04
		HD3	1.17
		HE2	2.55
		HG2	0.73
		HN	7.18
		NH	119.72
19	MET	CA	58.69
		CB	32.19
		CG	31.13
		HA	3.38
		HB2	1.10
		HB3	2.24
		HG2	1.53
		HN	7.40
		NH	117.72
20	HIS	CA	60.67
		CB	26.80
		HA	2.59
		HB2	1.29
		HB3	1.64
		HD1	8.16
		HD2	1.37
		HE1	1.48
		HN	6.29
		NH	115.10
21	GLN	CA	59.56
		CB	28.09
		CG	31.50
		HA	3.75
		HB2	1.36
		HG2	0.05
		HE21	1.70
		HN	7.56
		NH	119.44
22	GLU	CA	58.51
		CB	29.39
		CG	36.51
22	GLU	HA	3.81
		HB2	1.71
		HG2	1.94
		HG3	2.12
		HN	7.68
		NH	117.73
23	MET	CA	58.00
		CB	33.76
		CG	30.17
		HA	4.18
		HB2	1.76
		HG2	2.43
		HN	7.75
		NH	117.02
24	LEU	CA	56.31
		CB	42.45
		CG	27.19
		CD1	27.43
		CD2	22.51
		HA	4.53
		HB2	1.77
		HB3	2.15
		HG	1.68
		QD1	1.17
25	LYS	QD2	1.57
		HN	8.20
		NH	116.11
		CA	60.27
		CB	30.68
		CD	29.15
		CE	42.57
25	LYS	CG	25.38
		HA	4.12
		HB2	2.04
		HB3	2.32
		HD2	1.80
		HE2	3.11
		HG2	1.37
25	LYS	HN	7.73

Residue number	Residue type	Atom	Chemical shift (ppm)
25	LYS	NH	113.16
26	ASP	CA	53.73
		CB	41.43
		HA	5.01
		HB2	2.90
		HB3	3.00
		HN	8.49
		NH	118.98
27	CYS	CA	56.53
		CB	33.50
		HA	5.29
		HB2	2.52
		HB3	2.64
		HN	8.90
		NH	124.21
28	LYS	CA	57.40
		CB	31.60
		CD	29.24
		CE	41.86
		CG	25.11
		HA	3.80
		HB2	1.82
		HB3	1.93
		HD2	1.68
		HE2	2.97
		HG2	1.37
		HN	8.26
		NH	115.97
29	LYS	CA	58.40
		CB	32.55
		CD	29.06
		CE	42.64
		CG	26.06
		HA	4.22
		HB2	2.39
		HB3	2.44
		HD2	2.15
		HE2	3.39
		HE3	3.49

Residue number	Residue type	Atom	Chemical shift (ppm)
29	LYS	HG2	2.03
		HN	7.74
		NH	117.30
30	CYS	CA	54.50
		CB	39.49
		HA	4.87
		HB2	1.21
		HB3	2.78
		HN	6.54
		NH	111.12
31	HIS	CA	52.56
		CB	25.25
		HA	3.14
		HB2	0.98
		HB3	1.01
		HD1	8.84
		HD2	0.78
		HE1	1.22
		HN	7.22
		NH	116.84
32	GLU	CA	59.36
		CB	29.81
		CG	35.96
		HA	3.82
		HB2	1.88
		HB3	1.89
		HG2	2.12
		HN	8.28
		NH	125.93
33	ASN	CA	52.16
		CB	38.87
		HA	4.78
		HB2	2.35
		HB3	2.77
		HD21	6.88
		HD22	7.36
		HN	8.29
		NH	114.91
		ND2	112.90

Residue number	Residue type	Atom	Chemical shift (ppm)	Residue number	Residue type	Atom	Chemical shift (ppm)
34	GLY	CA	43.26	39	LYS	HD2	1.60
		HA	3.49			HE2	2.94
		HN	6.72			HG2	1.34
		NH	107.04			HN	8.50
36	GLY	CA	43.58			NH	130.56
		HA	3.46	40	ASP	CA	53.95
		HN	4.02			CB	39.62
		NH	109.13			HA	4.53
37	LYS	CA	56.41			HB2	2.60
		CB	31.97			HN	8.48
		CD	27.99			NH	116.28
		CE	42.27	41	PHE	CA	62.04
		CG	24.75			CB	39.49
		HA	4.03			HA	3.41
		HB2	1.63			HB2	2.85
		HD2	1.89			HD2	6.64
		HE2	3.18			HE2	6.95
		HE3	3.24			HZ	7.46
		HG2	1.62			HN	7.53
		HN	8.03			NH	119.03
		NH	116.69	42	GLY	CA	44.12
38	ILE	CA	61.22			HA2	3.93
		CB	39.07			HA3	4.32
		CD1	15.42			HN	6.02
		HA	3.60			NH	114.32
		HB	1.10	43	LYS	CA	59.45
		HG1	-0.38			CB	33.44
		HG2	0.46			CD	29.21
		QD1	-0.13			CE	41.80
		HN	10.11			CG	22.88
		NH	129.04			HA	2.57
39	LYS	CA	59.33			HB2	1.94
		CB	31.89			HD2	1.29
		CD	29.13			HE2	2.30
		CE	42.05			HE3	2.38
		CG	24.49			HG2	-1.26
		HA	3.85			HG3	-0.72
		HB2	1.65			HN	8.82
		HB3	1.70			NH	122.15

Residue number	Residue type	Atom	Chemical shift (ppm)	Residue number	Residue type	Atom	Chemical shift (ppm)
44	ASP	CA	57.30	48	LYS	CG	24.35
		CB	39.41			HA	3.63
		HA	3.42			HB2	1.45
		HB2	2.28			HD2	1.32
		HN	7.86			HD3	1.39
		NH	116.18			HE2	2.69
45	TRP	CA	62.10			HG2	0.95
		CB	31.44			HG3	1.11
		HA	3.71			HN	7.02
		HB2	2.93			NH	118.59
		HB3	3.24	49	THR	CA	66.62
		HD1	7.20			CB	69.50
		HE1	7.91			CG2	21.77
		HE3	7.73			HA	3.72
		HZ2	7.91			HB	3.17
		HZ3	7.96			QG2	1.40
		HH2	7.45			HN	8.21
		HN	8.32			NH	115.65
		NH	121.46	50	CYS	CA	58.57
46	ALA	CA	55.05			CB	35.65
		CB	18.82			HA	4.17
		HA	4.50			HB2	2.41
		QB	1.18			HB3	3.14
		HN	8.56			HN	8.21
		NH	122.32			NH	121.76
47	HIS	CA	55.37	51	LYS	CA	58.58
		CB	26.30			CB	31.26
		HA	2.55			CD	29.45
		HB2	1.40			CE	41.11
		HB3	1.55			CG	24.93
		HD1	9.46			HA	2.83
		HD2	1.01			HB2	0.99
		HE1	1.42			HD2	0.86
		HN	7.47			HE2	2.58
		NH	117.60			HG2	0.99
48	LYS	CA	58.79			HG3	1.14
		CB	32.57	52	GLY	CA	47.09
		CD	29.17			HA2	3.43
		CE	41.84			HA3	3.83

Residue number	Residue type	Atom	Chemical shift (ppm)
52	GLY	HN	6.25
		NH	104.89
53	CYS	CA	58.50
		CB	28.69
		HA	4.34
		HB2	2.31
		HB3	3.72
		HN	6.95
		NH	121.93
54	HIS	CA	55.31
		CB	28.43
		HA	3.76
		HB2	1.36
		HB3	1.50
		HN	5.96
		NH	115.59
55	THR	CA	66.43
		CB	68.88
		CG2	20.79
		HA	3.58
		HB	4.06
		QG2	1.06
		HN	7.63
		NH	113.07
56	GLU	CA	59.44
		CB	29.91
		CG	36.01
		HA	3.98
		HB2	2.17
		HG2	2.32
		HN	7.70
		NH	123.51
57	LEU	CA	55.43
		CB	41.69
		CD1	22.25
		CG	27.50
		HA	4.35
		HB2	1.77
		HB3	2.01
57	LEU	HG	2.29
		QD1	1.22
		HN	8.63
		NH	115.70
58	LYS	CA	57.23
		CB	29.00
		CE	42.49
		CG	25.04
		HA	3.73
		HB2	1.75
		HE2	3.05
59	LYS	HG2	1.38
		HN	7.62
		NH	115.34
		CA	55.69
		CB	37.66
		CD	29.93
		CE	42.62
60	GLY	CG	24.94
		HA	4.35
		HB2	1.71
		HB3	2.15
		HD2	2.05
		HE2	3.29
		HG2	1.65
		HG3	1.67
61	PRO	HN	7.71
		NH	119.01
		CA	42.59
		HA2	1.44
61	PRO	HA3	2.14
		HN	7.57
		NH	103.67
		CA	62.84
		CB	33.33
		CD	48.02
61	PRO	CG	29.14
		HA	4.02
		HB2	1.91

Residue number	Residue type	Atom	Chemical shift (ppm)	Residue number	Residue type	Atom	Chemical shift (ppm)
61	PRO	HD2	-0.59	67	CYS	CA	56.74
		HD3	2.57			CB	36.38
		HG2	1.93			HA	4.65
62	VAL	CA	61.92			HB2	1.50
		CB	34.22			HB3	2.08
		CG1	18.96			HN	6.28
		CG2	22.62			NH	114.34
		HA	4.36	68	HIS	CA	54.81
		HB	1.99			CB	26.44
		QG1	0.67			HA	2.69
		QG2	0.52			HB2	0.80
		HN	8.28			HB3	1.33
		NH	115.17			HD1	9.31
63	GLY	CA	44.88			HD2	0.89
		HA2	3.95			HE1	1.39
		HA3	4.72			HN	6.96
		HN	8.55			NH	116.26
		NH	111.23	69	LYS	CA	54.34
64	CYS	CA	58.72			CB	32.76
		CB	33.75			CD	28.90
		HA	5.01			CE	42.64
		HB2	2.81			CG	24.29
		HN	8.94			HA	4.35
		NH	119.96			HB2	1.70
65	THR	CA	62.09			HD2	1.55
		CB	68.17			HE2	2.92
		CG2	21.58			HE3	3.00
		HA	4.31			HG2	1.25
		HB	4.47			HN	7.72
		QG2	1.08			NH	124.02
		HN	8.10	70	LYS	CA	58.10
		NH	103.36			CB	33.55
66	ASP	CA	55.95			CD	29.29
		CB	41.19			CE	42.11
		HA	4.55			CG	24.87
		HB2	2.79			HA	3.96
		HB3	2.88			HB2	1.58
		HN	7.58			HB3	1.66
		NH	119.72			HD2	1.49

Residue number	Residue type	Atom	Chemical shift (ppm)
70	LYS	HE2	2.83
		HG2	1.24
		HN	7.90
		NH	129.11



2023

Marisa Raquel Martins de Brito Ferreira

Bacterial electrical routes: deciphering extracellular electron
transfer pathways in *Geobacter*

