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Thesis outline

The present work contributes to clarify decades of debate concerning the last step of the dissimilatory sulfate reduction pathway, the reduction of sulfite to sulfide. This work focused on proteins involved in the dissimilatory reduction of sulfite such as DsrAB, the soluble sulfite reductase, DsrC (crucial for DsrAB function), and DsrJ, a membrane-bound cytochrome c that belongs to DsrMKJOP membrane complex. In addition, we tried to find the physiological electron donor for the DsrAB enzyme.

This thesis begins with a short general introduction on sulfate reducing prokaryotes and their physiological properties. The second chapter contains the detailed characterization of the dissimilatory sulfite reductases of Desulfovibrio vulgaris and Archaeoglobus fulgidus as well as the description of the A. fulgidus DsrC mutants produced. This is followed by a third chapter concerning the reduction of sulfite by DsrAB and its interaction with DsrC, which revealed the important role of this small protein in the process. The fourth chapter includes studies to find the physiological electron donor for DsrAB testing several oxidoreductases such as pyruvate ferredoxin-oxidoreductase (Por), aldehyde oxidoreductase (Aor), the carbon monoxide dehydrogenase (CODH) and also NADH oxidase (NoxA-3). Chapter five covers the ongoing investigation about the heme protein DsrJ, a very special cytochrome that contains an unusual His/Cys distal axial heme c ligation, where several techniques were used to determine the nature and function of this ligation. Finally, the sixth chapter is a general conclusion that includes some considerations on the future perspectives of this work.
List of publications

A protein trisulfide couples dissimilatory sulfate reduction to energy conservation

* co-first authors

**Sulfur Isotope Effects of Dissimilatory Sulfite Reductase**

**Electron Transfer between the QmoABC Membrane complex and Adenosine 5’-Phosphosulfate Reductase**
Américo G. Duarte, **André A. Santos** and Inês A. Cardoso Pereira (2015) submitted to BBA – Bioenergetics – under review
Abstract

Life on Earth is extremely diverse, as organisms had to adapt to a panoply of different environmental niches. There are several mechanisms by which organisms grow in the absence of oxygen, one of which is the dissimilatory reduction of sulfate to sulfide, performed by sulfate reducing prokaryotes (SRP). This is thought to be one of the most ancient biological metabolisms to have developed on earth. In SRP sulfate is first imported to the cytoplasm where it is activated by Sulfate adenylyl transferase to adenosine 5′-phosphosulfate (APS) in an ATP consuming process. After, the adenylyl-sulfate reductase is the enzyme responsible for the reduction of APS to sulfite. The next step involves the reduction of sulfite to sulfide, but the mechanism involved in this process is not clear. There are two proposals that have been made for this step: an earlier one based on *in vitro* assays (the trithionate pathway) and a more recent one based on crystallographic information (DsrAB + DsrC pathway).

The work developed in this thesis aimed to contribute to the understanding this last step of sulfate reduction. The work was divided in three main tasks: i) the study of the interaction of DsrAB with DsrC in the reduction of sulfite, ii) the search for the physiological electron donor to DsrAB and iii) the characterization of the cytochrome c type DsrJ present in the DsrMKJOP complex.

The first task validated the DsrAB-DsrC pathway in which we proved that DsrC is crucial to the activity of sulfite reduction by DsrAB, being used as a co-substrate along with sulfite. Our work showed that the product of sulfite reduction by DsrAB is a DsrC trisulfide. This implies that in the energy conservation accomplished by the dissimilatory reduction of sulfide, four electrons are pooled from the reduced
menaquinone pool through the DsrMKJOP membrane complex to reduce the DsrC-trisulfide.

In the second task we aimed to disclose the physiologic electron donor of the first two electrons needed by DsrAB for the reduction of sulfite. To access this question we used three ferredoxin oxidoreductases (Aldehyde oxidoreductase, Pyruvate ferredoxin oxidoreductase and Carbon monoxide dehydrogenase) and one NADH oxidase (recombinant NoxA-3). These candidates were used in *in vitro* activity assays with DsrAB/sulfite and also in protein-protein interaction studies (surface plasma resonance). None of the candidates tested demonstrated to interact with DsrAB, but further experiments have to be carried out to discard them completely as electron donors.

The last task involved the analysis of the distal axial heme ligands of the DsrJ subunit of the DsrMKJOP complex, and was a continuation of previous work developed by Grein and coworkers. DsrJ is a protein that contains three heme binding motifs in which the corresponding ligands are proposed to be Histidine/Histidine, Methionine/Histidine and the unusual Cysteine/Histidine. Here we generated several variations on the *dsrJ* gene from *Allochromatium vinosum* in the potential distal axial ligands and analyzed these variants by several techniques: Electron paramagnetic resonance (EPR), Resonance Raman (RR), Nuclear magnetic resonance (NMR), Mass spectrometry (MS) and UV-visible. Although we generated more than ten variants, we were not able to identify the correct distal axial heme ligand for the three hemes present in the DsrJ protein. These results suggested that: i) there might be something during the protein purification that can bind to the modified heme in the absence of correct distal axial ligand or ii) that the protein is not correctly folded due to the absence of the other subunits of the membrane complex DsrMKJOP, although by MS the recombinant
protein and the tested mutants have the correct weight including three hemes.

The work presented here demonstrates the crucial role of the DsrC protein in DsrAB sulfite reduction, leading to energy conservation by the membrane complex DsrMKJOP. On the other hand, work on finding the physiological electron donor for the first two electrons needed by DsrAB has to be continued as it is extremely important to fully understand the dissimilatory sulfite reduction mechanism. The role of the DsrMKJOP complex, and more specifically of DsrJ, remains a mystery to be solved for both SRP and sulfate oxidizing bacteria (SOB), where it was proven to be essential for the oxidation of sulfur globules.
Sumário

A vida na Terra é extremamente diversificada, principalmente devido à adaptação de micro-organismos a diferentes nichos ambientais. Existem vários mecanismos pelos quais os micro-organismos conseguem obter energia na ausência de oxigénio, sendo um deles a redução dissimilativa do sulfato a sulfureto, realizada por organismos procariontes redutores de sulfato (SRP). Este poderá ser um dos mecanismos biológicos de obtenção de energia mais antigos, tendo aparecido muito antes da disponibilidade de oxigénio na atmosfera. Nestes organismos o sulfato é primeiro importado para o citoplasma, onde é ativado pela sulfato adenilil-transferase a adenosina 5'-fosfosulfato (APS), num processo com consumo de ATP. Seguidamente, a APS reductase é a enzima responsável pela redução de APS a sulfito. O próximo passo envolve a redução de sulfito de sulfureto, mas o mecanismo envolvido neste processo não é claro. Existem duas propostas para esta etapa: uma baseada em ensaios in vitro (a via do tritionato) e outra, mais recente, com base em informações cristalográficas (via da DsrAB + DsrC).

O trabalho desenvolvido nesta tese teve como objetivo contribuir para a compreensão deste passo da redução do sulfato. O trabalho foi dividido em três tarefas principais: i) o estudo da interação da DsrAB com DsrC na redução de sulfito, ii) a pesquisa do dador de eletrões fisiológico para DsrAB e iii) a caracterização de DsrJ, um citocromo tipo c, presente no complexo membranar DsrMKJOP.

A primeira tarefa validou a via DsrAB-DsrC na qual se provou que a DsrC é crucial para a atividade de redução de sulfito pela DsrAB, sendo utilizada como um co-substrato juntamente com sulfito. O nosso trabalho mostrou que o produto de redução do sulfito pela DsrAB é um
trissulfureto associado à DsrC. Este facto implica que, na conservação de energia associada à redução dissimilativa de sulfato, quatro eletrões serão provenientes da menaquinona membranar através do complexo DsrMKJOP reduzindo o DsrC-trissulfureto.

A segunda tarefa teve como objetivo descobrir o doador fisiológico dos dois primeiros eletrões requeridos pela DsrAB para a redução de sulfito. Para tal, foram utilizadas três ferredoxina oxidoreductases (aldeído oxidoreductase, piruvato ferredoxina oxidoreductase e desidrogenase de monóxido de carbono) e uma NADH oxidase (NOXA-3). Estes possíveis dadores foram testados em ensaios de atividade in vitro com DsrAB/sulfito e também em estudos de interação proteína-proteína (Ressonância plasmónica de superfície). Nenhum dos possíveis dadores testados demonstrou interagir com DsrAB, porém mais testes deverão ser realizadas de modo verificar estas conclusões.

A última tarefa envolveu a análise dos ligandos axiais distais dos hemos presentes na subunidade DsrJ do complexo membranar DsrMKJOP, tendo este trabalho sido uma continuação de trabalho anterior desenvolvido por Grein e colaboradores. A DsrJ é uma proteína que contém três motivos de ligação a hemos tipo c, nos quais os ligandos propostos são Histidina/Histidina, Metionina/Histidina e uma invulgar Cisteína/Histidina. De modo a verificar a identidade destes ligandos, produzimos vários mutantes da proteína DsrJ de *Allochromatium vinosum* com alterações nos potenciais ligandos axiais distais e analisámos-o através de várias técnicas: Ressonância paramagnética eletrônica (EPR), Ressonância de Raman (RR), Ressonância magnética nuclear (RMN), espectrometria de massa (MS) e UV-visível. Embora tenhamos gerado mais de dez variantes, não foi possível identificar os ligandos axiais distais dos hemos presentes na proteína DsrJ. Estes resultados sugerem que: i) poderá existir algo que
durante a purificação das proteínas se ligue aos hemos modificados na ausência do ligando axial distal original ou ii) que as proteínas recombinantes poderão não estar corretamente estruturadas devido à ausência das outras subunidades do complexo de membrana DsrMKJOP, embora pela técnica de MS a proteína recombinante e os mutantes testados tivessem a massa molecular correta incluindo três hemos.

O trabalho aqui apresentado demonstra o papel crucial da proteína DsrC na redução do sulfito pela DsrAB, ligando este mecanismo à conservação de energia através da interação com o complexo membranar DsrMKJOP. Por outro lado, o objetivo de encontrar o doador fisiológico de eletrões para os dois primeiros eletrões necessários pela DsrAB não foi conclusivo e deverá ser continuado, uma vez que é extremamente importante para compreender o mecanismo de redução dissimilativa do sulfito. O papel do complexo membranar DsrMKJOP-mais especificamente da DsrJ- permanece inconclusivo, sendo necessário ser resolvido tanto para SRP como para as bactérias oxidantes de sulfato, onde o papel desta proteína foi demonstrado ser essencial para a oxidação dos glóbulos de enxofre.
List of abbreviations

AOR  Aldehyde oxidoreductase
ATP  Adenosine triphosphate nucleotide
APS  Adenosine-5'-phosphosulfate
ApsBA Adenosine-5'-phosphosulfate reductase
aSiR Assimilatory sulfite reductase
CODH Carbon monoxide dehydrogenase
D. Desulfovibrio
D. vulgaris Desulfovibrio vulgaris Hildenborough
Dsr Dissimilatory sulfite reductase
DTT Dithiothreitol
e' electron
EPR Electron paramagnetic resonance
Fdx Ferredoxin
Fe-S iron-sulfur cluster
g EPR g-value
GSA gel shift assay
Hdr heterodisulfide reductases
HEPES N-(2-hydroxyethyl)-N'-2-ethanesulfonic acid
Hmc high molecular mass cytochrome complex
HPLC high performance liquid chromatography
PPi inorganic pyrophosphate
IPTG isopropyl-β-D-thiogalactoside
LGT lateral gene transfer
MALDI-TOF matrix-assisted laser desorption ionization/time-of-flight
MalPEG methoxy-polyethylene glycol maleimide
MIC Microbially influenced corrosion
MS Mass spectrometry
MQ menaquinone
MQH₂ menaquinol
Nar  nitrate reductase
Nrf  nitrite reductase
Nhc  nine-heme cytochrome complex
NMR  Nuclear magnetic resonance
NoxA-3 NADH oxidase from A. Fulgidus
Ohc  octaheme cytochrome complex
PAGE  polyacrylamide gel electrophoresis
PAPS  phosphoadenosine phosphosulfate
Qmo  quinone-interacting membrane oxidoreductase complex
Rnf  Rhodobacter nitrogen fixation complex
RR  Resonance Raman
Sat  Sulfate adenylyl transferase
SDS  sodium dodecyl sulfate
Sox  sulfur oxidation enzymes
SiR  sulfite reductases
SiRHP  aSiR siroheme-containing subunit
SiRFP  aSiR flavin-containing protein
PFOR  Pyruvate ferredoxin oxidoreductase
SOB  sulfur oxidizing bacteria
SPR  Sulfate reducing prokaryotes
SRP  Surface plasmon resonance
Tricine  N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine
Tris  tris(hydroxymethyl)-aminomethane
UV  ultra-violet
Wt  wild type
Chapter 1

Introduction
Introduction

Sulfur is one of the most versatile nonmetallic elements on Earth, due to its broad range of oxidation states that go from -2 (sulfide) to +6 (sulfate). Sulfur can be found in minerals such as pyrite (FeS₂) or gypsum (CaSO₄) or in biological compounds like amino acids, nucleosides, protein cofactors, co-enzymes, vitamins, metabolites, sulfolipids and hormones (Ehrlich & Newman, 2008). Sulfur compounds can also be oxidized or reduced microbiologically and the sulfur cycle is crucial for the maintenance of the life on Earth, being connected to other cycles such as that of carbon and nitrogen (Muyzer & Stams, 2008). As a major biogeochemical cycle in nature (Luptakova, 2007), it has been shown that 75% of the crustal sulfur has been cycled biologically and that per year 4-5×10¹² kg of sulfate is cycled by living cells (Skyring & Donnelly, 1982).

The microorganisms capable of reducing sulfate are known as sulfate reducing prokaryotes (SRP), which can be present in a wide variety of anaerobic environments (Peck et al., 1982). SRP are more abundant in marine settings, the biggest reservoir of sulfate (Vairavamurthy et al., 1995), where it is the second most abundant anion after chloride. An historical overview of research in SRP is available in Rabus et al., 2006. Interest in sulfate reduction began after 1864 when Meyer realized that the remarkable concentrations of hydrogen sulfide in aquatic systems were produced biologically through the reduction of sulfate (Meyer, 1864). In 1895 the first sulfate-reducing bacterium was isolated and named *Spirillum desulfuricans* by Beijerinck’s co-workers. The first real evidence for a sulfur reduction mechanism as the main energy source for microbial growth was found by Pelsh in 1936, with *Desulfuromonas acetoxidans* being the first organism grown by sulfur
Chapter 1

reduction (Pelsh, 1936). The initial breakthrough studies into the biochemistry of the SRP occurred mainly during the 1950s and 1960s. Desulfovibrio was then identified as the first anaerobe with a cytochrome, which previously was only believed to be associated with aerobic respiration. In 1956 Postgate discovered a green protein that reduced sulfite to sulfide and named it desulfoviridin (Postgate, 1956). Later, in 1958, Lipmann reported on the main differences between the assimilatory and dissimilatory sulfate reduction pathways (Lipmann, 1958). The dissimilatory sulfate reduction was than showed by Jørgensen and coworkers to be responsible for the mineralization of about half of the organic carbon in marine sediments (Jørgensen & Fenchel, 1974). The 1980s were the decade for the revolution on understanding the enzymatic reactions and bioenergetics of sulfate reduction. ATP balances with hydrogen were accurately measured with chemostat studies (Badziong & Thauer, 1978, Nethejaenchen & Thauer, 1984) and three different types of hydrogenases ([Fe-Fe], [Ni-Fe] and [Ni-Fe-Se]) were detected (Huynh et al., 1984, Rieder et al., 1984, Fauque et al., 1988). Another essential development was the beginning of molecular studies in Desulfovibrio species that made possible the analysis in more detail of several crucial metabolic enzymes. The first 16S rRNA comparative analysis of a sulfate reducing bacteria was performed by Oyaizu and Woese (Oyaizu et al., 1985). They exposed the relationships of Desulfovibrio desulfuricans with Myxococcus and photosynthetic purple bacteria. This revolutionized the phylogenetic organization of sulfate reducing prokaryotes. In the 1990s, improvements in the study of single proteins, genes and environmental impact led to a better understanding of SRP at metabolic and physiologic levels. In 1997, the first completely sequenced genome of an SRP from the archaeon Archaeoglobus fulgidus, was achieved by Klenk and collaborators (Klenk et al., 1997). The genome of Desulfovibrio vulgaris
Hildenborough, one of the most studied SRP, was fully sequenced in 2004 by Heidelberg and coworkers (Heidelberg et al., 2004).

Sulfate can be anaerobically respired using gaseous hydrogen as the energy source or heterotrophically when using organic substrates like pyruvate, formate, lactate, malate, succinate or ethanol (Muyzer & Stams, 2008). SRP are divided into two major groups regarding the degradation of organic compounds: those that degrade organic compounds incompletely to acetate and those that degrade it completely to carbon dioxide (Muyzer & Stams, 2008). They are also known for their ability to grow on diverse substrates besides the simple ones mentioned above. These substrates include: sugars, amino acids, one-carbon compounds (such as methanol, carbon monoxide and methanethiol), benzoate and phenol, aromatic hydrocarbons, short and long-chain alkanes (Muyzer & Stams, 2008). Moreover, many SRP are able to grow by disproportionating thiosulfate, sulfite and sulfur, resulting in the formation of sulfate and sulfide (Bak & Pfennig, 1987, Bottcher et al., 2005). Being anaerobes, SRP were considered to be unable to use oxygen as energy source, but many can tolerate oxygen for periods of time, and include terminal oxidase genes in their genomes (Dolla et al., 2007). Lobo et al demonstrated that, from a variety of SRP that can tolerate oxygen, D. desulfuricans ATCC 27774 has the ability to grow in a lactate/nitrate medium (absence of sulfur compounds) under nearly atmospheric oxygen levels (Lobo et al., 2007). SRP are distributed in a variety of environments ranging from marine to freshwaters, hot environments (such as hydrothermal vents and mud volcanoes), extreme pH habitats (acid-mine drainage sites with pH as low as 2-3) and soda lakes (pH high as 10) (Rabus et al., 2015). They can also be part of microbial consortia with microorganisms such as methanotrophic archaea or with sulfur oxidizing bacteria (Muyzer & Stams, 2008).
SRP can be found in four bacteria phyla (Proteobacteria - class Deltaproteobacteria, Nitrospirae, Firmicutes and Thermodesulfobacteria) and two archaeal phyla (Euryarchaeota and Crenarchaeota) (Müller et al., 2015). These groups of organisms have been taxonomically characterized by several techniques such as cultivation (which can detect less than 1% of the total microorganisms), analysis of the phospholipid fatty acids and 16S ribosomal RNA (rRNA) (Muyzer & Stams, 2008). Comparative analysis of the dsrAB genes is a commonly used technique to identify novel lineages of SRP (Wagner et al., 2005, Hansel et al., 2008, Loy et al., 2008, Müller et al., 2015). The DsrAB enzymes are assumed to be ancient, with an ancestor present before the division of the domains Bacteria and Archaea (Wagner et al., 1998, Dhillon et al., 2005, Loy et al., 2009). The distribution of the dsrAB genes among prokaryotes is now known to have involved divergent speciation, functional diversification and lateral gene transfer (LGT) (Loy et al., 2008). The dsrAB genes present in the genus Archaeoglobus, Thermodesulfobacterium and in few low-GC content Gram-positive bacteria of the phylum Firmicutes have been shown to derive from Deltaproteobacteria donor, proving the existence of LGT even across domains (Larsen et al., 1999, Klein et al., 2001, Stahl et al., 2002, Zverlov et al., 2005). This makes the comparative dsrAB gene analysis an important technique for the detection of new SRP and sulfur oxidizing microorganisms even in environmental samples. However, it must be noted that other organisms besides SRP and sulfur oxidizing bacteria may also contain dsrAB genes. This includes sulfur disproportionators and organisms that reduce sulfite, thiosulfate or organosulfonates.

Sulfur species can be easily oxidized to sulfate in the presence of oxygen, with the levels of atmospheric oxygen being crucial for this process. It is known that the atmospheric oxygen levels only arose
around 2.45 billion years ago, after the Great oxygenation event (Holland, 2006). This event increased marine sulfate to the levels present of today (~28mM) (Canfield et al., 2000). The understanding of the evolution of the sulfate reservoir, as a proxy for atmospheric oxygen levels, can be achieved by measuring the ratio of stable sulfur isotopes present in sulfur-bearing minerals. Sulfur has the ability to exist in 4 stable isotopes: $^{32}$S and $^{34}$S (the two most abundant), and $^{33}$S and $^{36}$S (minor isotopes) (Johnston, 2011). Sulfur isotope discrimination has been used to study dissimilatory sulfate reduction performed by SRP, as this metabolic process induces a large mass-dependent fractionation between sulfate and sulfide that is preserved in geological records (Johnston, 2011). This occurs because SRP favors light sulfur isotopes ($^{32}$S) instead of heavier ones ($^{34}$S) (Wing & Halevy, 2014). This can be explained by the fact that S-O bonds of lighter isotopes are easier to brake during sulfate reduction (Lyons & Gill, 2010). Through isotope analysis Shen and coworkers found evidence that microbial sulfate respiration had evolved 3.47 Gyr ago (Shen et al., 2001). Culture experiments with SRP were one of the first metabolisms to be isotopically characterized (Thode H. et al., 1951). Through these experiments, isotope fractionation has been inversely correlated with cell sulfate reduction rates, and directly with extracellular concentrations of sulfate (Harrison & Thode, 1958, Kaplan & Rittenberg, 1964, Chambers et al., 1975, Leavitt et al., 2013).

Sulfate respiration is of great importance for the biogeochemical sulfur cycle, and sulfur isotope fractionation has been used for decades as a proxy for atmospheric oxygenation leading to a better understanding of Earth history (Rabus et al., 2015).
1.1 Biological and economic impact of SRP

SRP can have very important biotechnological applications. Recently, Rabus and coworkers reviewed on the major biotechnological/environmental impact of SRP in the last 20 years (Rabus et al., 2015). SRP are known to be extremely important for bioremediation of heavy and radioactive metals and organic compounds (oxidation of monoaromatic hydrocarbons, dehalorespiration and nitroaromatic respiration) and for the reduction of azo dyes (Barton & Fauque, 2009, Rabus et al., 2015). Due to their high tolerance to metal contamination, they are used for the removal of heavy metals from acidic mine drainage waters (Martins et al., 2009, Martins et al., 2011, Sánchez-Andrea et al., 2011, Sánchez-Andrea et al., 2014). Moreover, SRP are used in waste water and off gas treatments to remove metals, sulfate and CO (in off gas) (Rabus et al., 2015). There are three processes accomplished by SRP that allows them to be used for bioremediation: growth by sulfate reduction (precipitation of metals that react with sulfide), uranium reduction and mercury methylation (Rabus et al., 2015). On the other hand, SRP are associated in major economic and health issues. SRP are implicated in the biocorrosion of ferrous metals, corrosion of concrete and stonework and also have a major impact on the petroleum industry (Barton & Fauque, 2009, Rabus et al., 2015). Microbially influenced corrosion (MIC) by SRP can be accomplished through three mechanisms: the chemical attack of iron by hydrogen sulfide, the use of the cathodic hydrogen (generated through slowly chemical oxidation of Fe$^0$) or by directly taking up of the electrons from the metallic iron (Beech & Sunner, 2007, Rabus et al., 2015). In the oil industry SRP cause problems not only in pipeline corrosion but also because of their growth in crude oil and the production of hydrogen sulfide in crude oil (a process called souring of oil fields) that
contaminates this expensive and extremely important raw material for our society (Voordouw, 2011). Concerning human health, it was long demonstrated that SRP are present in the human gut (Gibson et al., 1988). SRP are present in all humans and, although not being part of the dominant intestinal flora, are now known as belonging to the human microbiome (Carbonero et al., 2012). These SRP have been implicated with the onset or perpetuation of chronic inflammatory bowel diseases such as Crohn’s disease and ulcerative colitis (Loubinoux et al., 2002). However, it remains to be revealed if SRP are the cause of the inflammatory process or if they are opportunistic colonizers of the inflamed area (Verstreken et al., 2012). SRP have been also implicated with clinical severity of human periodontitis (Langendijk et al., 2000). Furthermore, SRP have been isolated from profound abscesses (abdominal and brain), urine and blood (Tee et al., 1996, McDougall et al., 1997, La Scola & Raoult, 1999, Loubinoux et al., 2000). Recently it was hypothesized that these SRP can also be involved in the development of autism spectrum disorders (Weston et al., 2015). It is difficult to grow and isolate SRP specimens from clinical samples, but this can be overcome with molecular techniques for SRP identification such as 16S rRNA gene sequencing or the identification of specific genes like apsBA or dsrAB (Nakao et al., 2009, Müller et al., 2015).
1.2 Sulfate reduction mechanism: Assimilatory vs Dissimilatory pathways

There are two biological pathways for sulfate reduction: it can be assimilated and transformed into several biological molecules, a capacity found in prokaryotes, fungi and plants or it can be dissimilated (a respiratory process) where it acts as electron acceptor. Sulfur compounds of intermediate oxidation state can also be disproportionated by some SRP and other highly specialized-bacteria. In this case, elemental sulfur, sulfite or thiosulfate function simultaneously as electron donor and acceptor, for the generation of energy (Tang et al., 2009). The dissimilatory mechanism is considered a true respiratory process accomplished by several prokaryotes (Bacteria and Archaea). In both assimilatory and dissimilatory mechanisms sulfate is reduced in the cytoplasm. In the assimilatory pathway sulfate is imported by a sulfate permease (SulT family in prokaryotes and SulP/SLC26 family in eukaryotes) (Pilsyk & Paszewski, 2009), and in the dissimilatory pathway by electroneutral proton-anion symport in freshwater microorganisms (Cypionka, 1987) or sodium ion gradient produced by Na⁺ antiport in marine strains (Kreke & Cypionka, 1994). Thermodynamically, the redox potential for the sulfate/bisulfite couple is -516 mV, too low to allow reduction by a physiological electron donor such as ferredoxin (~-400 mV) or NADH (~-320 mV). Therefore sulfate needs to be activated before it can be reduced (Thauer et al., 2007). Once inside the cytoplasm sulfate is activated by Sulfate adenylyl transferase (Sat) with the consumption of ATP molecules to adenosine 5’-phosphosulfate (APS) and inorganic pyrophosphate (PPI) (Ullrich et al., 2001). This results in an increase of the redox potential from -516 mV for the sulfate/sulfite couple to -60 mV for the APS/sulfite couple (Thauer et al., 2007). PPI must be further hydrolyzed to 2 Pi to pull the reaction to the
direction of APS (Ehrlich & Newman, 2008). This hydrolysis can be accomplish by soluble inorganic pyrophosphatases (most cases) or, in some SRP, it can be performed by membrane-associated proton translocating pyrophosphatases, coupling this reaction to energy conservation (Pereira et al., 2011). The differences between assimilatory and dissimilatory pathways start here (Figure 1.1). In the dissimilatory pathway APS is then reduced by adenylyl-sulfate reductase (ApsBA) to sulfite and adenosine monophosphate (AMP). In contrast, in most assimilatory pathways, APS is further phosphorylated to 3′-phosphoadenosine 5′-phosphosulfate (PAPS) by APS-kinase activity, which is only then reduced by PAPS reductase to sulfite. The final step is the reduction of sulfite to sulfide, which has a redox potential of

\[
\text{Sulfite} \rightarrow \text{Sulfide (H}_2\text{S)} \rightarrow \text{HS}^- \rightarrow \text{H}_2\text{S}
\]

Figure. 1.1. Schematic representation of the difference between the assimilatory and dissimilatory pathways for sulfate reduction. Blue – common pathway; Green – Dissimilatory pathway; Orange – Assimilatory pathway.
- 116 mV (Muyzer & Stams, 2008). In the assimilatory reduction, the aSiR is capable of reducing sulfite in one single step to sulfide, without the formation of other products (Crane & Getzoff, 1996). On the other hand, it was shown that in in vitro assays with DsrAB and sulfite, DsrAB produces a mixture of compounds with thiosulfate, thionitrite and sulfide being formed (Lee & Peck, 1971, Akagi, 1983).

1.3 Sulfite reductases

The assimilatory and dissimilatory pathways involve two forms of sulfite reductases (SiR): the assimilatory sulfite reductase (aSiR) and the dissimilatory sulfite reductase (DsrAB). The SiR are proteins thought to be present in LUCA (Last Universal Common Ancestor) which plays a crucial role in the reduction of sulfite to sulfide (Wagner et al., 1998, Castresana & Moreira, 1999, Larsen et al., 1999). These enzymes are not only considered ancient but crucial for the biologic conversions of sulfur compounds in the anoxic and extremely reduced primordial Earth (Wagner et al., 1998). The reduction of sulfite to sulfide and nitrite to ammonia are the only examples of single-step six-electron reductions found in nature (Stroupe & Getzoff, 2009). Through comparative studies it is known that among the SiR, the basic framework has been conserved. The motif Cys-X₅-Cys-X₅'-Cys-X₃-Cys is found to be highly conserved in all SiRs (Karkhoff-schweizer et al., 1995). This motif harbors the catalytic center, a unique cofactor that includes a siroheme (iron-sirohydrochlorin) bound to an iron-sulfur [4Fe-4S] cluster (Figure 1.2) (Crane et al., 1997). It has been found that the biosynthetic pathway for siroheme is more ancient than that of cytochromes and are closely related to cobalamin (vitamin B12) (Stroupe & Getzoff, 2009, Lobo et al.,
2012). This supports the hypothesis of an ancestral origin for sulfite reduction, in early prokaryotes period, long before aerobic respiration. Moreover, the analysis of the structure and sequence identity between the aSiR siroheme-containing subunit (SiRHP) with the α and β subunits of DsrAB indicates that they are phylogenetically related and the aSiR diverged from a gene-duplication event followed by gene fusion of an ancestral sulfite reductase gene present in a very early life form (Crane et al., 1995, Oliveira et al., 2008, Rabus et al., 2015). The siroheme has a structural organization that allows it to push electrons into the substrate. On the other hand, the distorted geometry and carboxylate side chains of the siroheme promotes the stabilization of an high effective proton concentration overhead the substrate which indirectly pulls electrons from the substrate facilitating its reduction (Crane & Getzoff, 1996). Among SiR there are several variances in the oligomeric state, the number of prosthetic groups and the electron delivery system (Schiffer et al., 2008).
**Assimilatory sulfite reductase.** The aSiRs are widespread among prokaryotes, fungi and plants. The aSiR from *E. coli* is a heavy oligomer (> 700 kDa) containing eight copies of a flavin-containing protein (SiRFP; 66 kDa) and four copies of the siroheme-containing hemoprotein (SiRHP; 64 kDa) (Siegel *et al.*, 1974, Crane & Getzoff, 1996). In this holoenzyme the SiRFPs are responsible for accepting electrons from NADPH and the SiRHPs (Figure 1.3) contain the catalytic centers that accept the electrons from the flavoprotein subunits and reduce the substrate (Figure 1.4 (B)) (Crane & Getzoff, 1996). The first crystallographic structure of an aSiR from *Escherichia coli* (PDB code 1AOP) was solved in 1995 by Crane and coworkers (Crane *et al.*, 1995). This structure showed that SiRHP is formed by a tri-lobed protein with a pseudo two-fold symmetry. A siroheme-[4Fe-4S] cofactor is present in the junction of the three domains (Crane *et al.*, 1995). On the other hand, the aSiR from *Arabidopsis thaliana* is a monomeric protein containing only the SiRHP unit which can accept electrons from an NADPH:ferredoxin reductase or photosystem I through a ferredoxin (Figure 1.4 (A)) (Nakayama *et al.*, 2000). It is well documented that aSiR
can catalyze the six-electron reduction of sulfite to sulfide in one single-step (Crane & Getzoff, 1996, Smith & Stroupe, 2012)

**Figure. 1.4.** Schematic representation of the electron donors of the sulfite reduction system in plants (A) and E. coli (B). Electrons are donated to plant SiR from Fd reduced by photosystem I (PS I) or the NADPH/FNR/Fd cascade. E. coli NADPH-SiR is a complex hemoflavoprotein with a subunit structure of αβ8: the α subunit is a flavoprotein containing FAD and FMN and the β subunit is a hemoprotein containing siroheme and a [4Fe–4S] cluster (Nakayama et al., 2000)
**Dissimilatory sulfite reductase.** The dsrAB genes are widespread among SRP and encode the proteins responsible for the reduction of sulfite in anaerobic respiration. It can also be present in several organisms that reduce thiosulfate, sulfite or organosulfonates, in syntrophic bacteria and organisms that disproportionate sulfur compounds (Simon & Kroneck, 2013). All dissimilatory sulfate organisms known to data have the dsrAB genes (Zverlov et al., 2005). The dsrAB genes can also be present in sulfite-reducing organisms such as *Desulfitobacterium*, *Desulfitibacter* and *Pyrobaculum* (Simon & Kroneck, 2013), sulfur-disproportionating bacteria, organosulfonate metabolizing organisms, and some sulfur-oxidizing bacteria (SOB), where the reversible DsrAB is essential for the oxidation of sulfur globules (Pott & Dahl, 1998, Loy et al., 2009). The DsrAB proteins are $\alpha_2\beta_2$ heterotetramers with molecular masses between 145 and 225 kDa (Rabus et al., 2006) and with the $\alpha$ subunit larger (about 50 kDa) than the $\beta$ subunit (typically 40 kDa). According to their characteristic UV-visible spectrum, DsrAB proteins were classically divided in four classes: desulforubidin (Lee et al., 1973), desulfofuscidin (Moura et al., 1988), P-582 (Trudinge.Pa, 1970) and desulfoviridin (Lee et al., 1973). Nowadays, it is known that these small changes in the UV-visible spectrum are not correlated with the significant structural differences to justify a division of the DsrAB proteins in classes, being referred only as DsrAB (Rabus et al., 2015). Although, in the case of desulfoviridin there is an extra peak in the UV-visible spectrum at the 630 nm area that reveals a different feature. This characteristic peak is due to the presence of a sirohydrochlorin, which is a siroheme without Fe (Figure 1.5). Another difference between Dsr proteins is that, in some cases (ex.: *D. vulgaris* and *D. desulfuricans*), the oligomeric state of the purified proteins is $\alpha_2\beta_2\gamma_2$. This third $\gamma$ protein was suggested to be a subunit of the desulfoviridin proteins. However, this protein corresponds to DsrC,
whose gene is not in the same transcriptional unit as the *dsrAB* genes, and their expression is not coordinated (Karkhoff-Schweizer *et al.*, 1993). This indicates that DsrAB and DsrC are two different proteins that, in the case of *D. vulgaris* and *D. desulfuricans*, can form a tight complex. The DsrC protein was proposed to be involved in the mechanism of sulfite reduction (Oliveira *et al.*, 2008) (see further discussion in chapter 1.4).

*Figure. 1.5. Scheme of sirohydrochlorin and siroheme*
Only with the determination of the crystal structure of DsrAB in 2008, from *D. vulgaris* (Oliveira *et al.*, 2008) and *A. fulgidus* (Schiffer *et al.*, 2008), was the cofactor composition of this enzyme truly elucidated (Figure 1.6). The structures showed that the DsrAB proteins only have two catalytic siroheme-[4Fe-4S] cofactors, one per each αβ unit, present in DsrB. There is also one siroheme (in the case of *A. fulgidus*) or sirohydrochlorin (in the case of *D. vulgaris*) per each DsrA subunit, but these are non-catalytic. In the case of the siroheme in *A. fulgidus*, there is a tryptophan residue blocking the possible sulfite binding site. This tryptophan is not conserved among all DsrAB proteins but it is conservatively exchange by a phenylalanine in the DsrA subunit. Moreover, in the DsrA subunit the amino acids surrounding the siroheme are different from those present in the catalytic cavity of DsrB subunit, forcing the negatively charged acetate and propionate groups to repel incoming anions (Schiffer *et al.*, 2008). Concerning the sirohydrochlorin in *D. vulgaris*, the absence of the Fe obviously precludes any catalytic activity and the positive electrostatic potential at the possible substrate channel that facilitates the entrance of the negatively charged sulfite.
(present in the catalytic center in DsrB) is absent in the DsrA subunits. Also, the existence of an arginine and a tryptophan near the sirohydrochlorin cavity in DsrA blocks its access by sulfite (Oliveira et al., 2008). Overall, this shows that the DsrAB proteins have evolved to lose the catalytic site in DsrA. A similar situation is observed for the assimilatory proteins where the second cofactor is no longer even present.

It was already referred that the DsrAB proteins do not reduced sulfite exclusively to sulfide. Several authors verified that the in vitro formation and relative proportion of the several sulfur products was connected with the concentration of electron donor (methyl viologen or hydrogenase) and the concentration of substrate (sulfite) (Akagi, 1995). They verified that higher concentrations of electron donor and low concentration of substrate led to production of more sulfide and less trithionate/thiosulfate, and that the opposite (less electron donor and high substrate concentrations) produced more trithionate than sulfide (Kobayashi.K et al., 1972, Kobayashi.K et al., 1974, Jones & Skyring, 1975, Drake & Akagi, 1977, Akagi, 1983, Akagi, 1995). These results led to the proposal of an alternative pathway for the dissimilatory reduction of sulfite. This pathway was named the trithionate pathway, where it was proposed that the DsrAB reduces sulfite to a mixture of thiosulfate, trithionate and sulfide. Further details of this mechanism were proposed by Parey and coworkers through the analysis of the *A. fulgidus* DsrAB crystal structure complexed with SO$_3^{2-}$, S$^2$, NO$_2^-$, CO and CN$^-$ (Parey et al., 2010) (Figure 1.7). In this proposal, they divided the process into three two-electron transfer steps, each one involving the acceptance of two protons and release of one H$_2$O molecule. The proposed mechanism starts with the bisulfite (the form of sulfite at pH 6) being reduced at the catalytic siroheme-[4Fe-4S] cofactor to form an S(II) intermediate. From
Here, two things can happen: this intermediate can react with more bisulfite forming thiosulfate that can further react with another bisulfite releasing trithionate, or it can be further reduced to an S(0) intermediate. Again, this S(0) intermediate can react with free bisulfide releasing thiosulfate, or it can be further reduced to sulfide as the end product (Parey et al., 2010).

![Diagram](image)

Figure 1.7. Scheme of the six-electron reduction of sulfite to trithionate, thiosulfate and sulfide. Only residues that directly interact with the siroheme iron-bound sulfur-oxygen adduct are noted. The process is subdivided into three two-electron transfer steps each accompanied by acceptance of two protons and release of one H₂O molecule. Trithionate and thiosulfate might be produced if the S(II) and S(0) intermediates are sufficiently long-living to be attacked by sulfite; these side reactions are supported by high sulfite concentrations that are unlikely to exist within the cell. (Parey et al., 2010)

Nevertheless it has been questioned whether this trithionate pathway is truly physiologically relevant. This pathway would require the
existence of trithionate and thiosulfate reductases in all SRP, which have not been shown to be present in all SRP (Pereira et al., 2011). There is another proposed mechanism by Oliveira and coworkers for dissimilatory sulfite reduction in which the DsrC protein is considered fundamental for the reduction of sulfite into the single product sulfide (Oliveira et al., 2008) (see chapter 1.4).

### 1.4 DsrC, the missing link in dissimilatory sulfite reduction

In 2008, Oliveira and coworkers revealed new insights into the pathway of sulfite reduction through the analysis of the structure of the *D. vulgaris* Hildenborough DsrAB in complex with DsrC. Here, they pointed out the crucial role of DsrC. DsrC was first described by Pierik in 1992 (Pierik et al., 1992) as a third subunit of the DsrAB enzyme from *Desulfovibrio vulgaris* Hildenborough which have a $\alpha_2\beta_2\gamma_2$ structure. In 1993, it was shown that these three genes were not co-regulated and additionally the *dsrC* gene was not present in the same operon as *dsrAB* genes (Karkhoff-schweizer et al., 1993). Other purified DsrAB proteins from SRP (ex.: *Pyrobaculum aerophilum* and *A. fulgidus*) do not contain DsrC, having a $\alpha_2\beta_2$ rearrangement, suggesting that DsrC protein is an independent protein rather than a third subunit of DsrAB (Dahl et al., 1993, Cort et al., 2001, Mander et al., 2005, Cort et al., 2008). It is established that DsrC belongs to TusE/DsrC/DsvC family (Venceslau et al., 2014). This family contains proteins with 12-14 kDa, with a helix-turn-helix (HTH) motif, and a highly conserved C-terminal arm. The HTH motif is associated with protein or DNA binding but can also be observed in protein-protein interactions (Aravind et al., 2005, Venceslau et al., 2014). The family can be divided in three major protein groups: DsrC, TusE and RspA (Venceslau et al., 2014). DsrC proteins have two
conserved cysteines in their C-terminal arm corresponding to the Cys$_B^{-}$X$_{10}$-Cys$_A$ motif, in which the Cys$_A$ is the penultimate amino acid in the C-terminal sequence and Cys$_B$ is eleven residues before (Figure 1.8). Accordingly to the NMR structure of *P. aerophilum* DsrC, the C-terminal is flexible, switching between the retrated and estended form (Cort *et al.*, 2001). A crystal structure of DsrC from *A. fulgidus* in the retrated form showed that the two conserved cysteines are not in close enough proximity to form a disulfide bond (Mander *et al.*, 2005). However, it is possible, in the presence of oxidizing agents, to produce a disulfite bond between Cys$_B$ and Cys$_A$ (Venceslau *et al.*, 2013).

The TusE proteins have only the conserved Cys$_A$ and the RspA (stands for regulatory sulfur-related proteins) have no conserved cysteines in the C-terminal arm, but have the HTH motif (Venceslau *et al.*, 2014). According to Venceslau *et al.* the *dsrC* gene is present in all organisms that have the *dsrAB* genes, but the reverse is not verified, which reinforces the hypothesis that DsrC is essential for the function of DsrAB (Venceslau *et al.*, 2014). In fact, the expression of DsrC is equal

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**Figure. 1.8.** Alignment of the DsrC C-terminal arm. DsrC from sulfate reducing organisms (1), organisms that reduce sulfite or thiosulfate (2) and sulfur oxidizing bacteria (3) are included. Strictly conserved residues are in black. The key DsrC CysA and CysB residues are highlighted in red.
or higher than that of other dissimilatory sulfate reduction proteins, suggesting an important role in cell metabolism (Wall et al., 2008). The structural homology of DsrC to the Tet repressor TetR (a DNA binding protein that regulates the expression of the TetA, enzyme responsible for tetracycline antibiotic resistance (Kisker et al., 1995)) suggests that DsrC could be involved in transcriptional regulation (Cort et al., 2001, Venceslau et al., 2014). This was supported by Grimm and coworkers which demonstrated that DsrC from A. vinosum is able to bind to a promoter region upstream of the dsrA gene (Grimm et al., 2010). On the other hand, the homology of DsrC with TusE supports the idea that DsrC could be implicated in sulfur related reactions (Ikeuchi et al., 2006, Oliveira et al., 2011, Venceslau et al., 2014). The TusE protein is involved in a sulfur-relay mechanism in which its terminal cysteine is decisive for the biosynthesis of thiouridine (Ikeuchi et al., 2006). This mechanism starts with the activation of the S-atom of cysteine by the cysteine desulfurase IscS which forms an enzyme-bound persulfide. After that, the sulfur is carried by three small proteins: TusA, TusBCD and TusE. In TusE, the last cysteine of the C-terminal arm forms a persulfide and transfers the sulfur to MnmA, which finally incorporates it into tRNA (Figure 1.9) (Ikeuchi et al., 2006, Shigi, 2014).

![Figure 1.9. Biosynthesis pathway of 2-thiouridine In E. coli, Tus proteins relay the persulfide sulfur of IscS to a modification enzyme, MnmA. In the green circle is represented the TusE protein receiving the sulfur from the TusD and transferring it to the MnmA (Shigi, 2014).](image-url)
DsrC proteins have already been implicated in two crucial pathways in SRP and SRO (Oliveira et al., 2008, Stockdreher et al., 2012). In the sulfate reduction pathway, DsrC is proposed to be decisive for the reduction of sulfite. It was shown by Oliveira et al (2008) (Figure 1.10) that the C-terminal arm of DsrC binds inside a pocket between the DsrA and DsrB subunits (Oliveira et al., 2008). This brings the Cys_A residue right next to catalytic active site allowing the involvement of Cys_A in catalysis. In the *D. vulgaris* structure, DsrC is covalently linked to the siroheme-[4Fe-4S], which is also observed in the structures of *D. norvegicum* and *D. gigas* DsrAB (Oliveira et al., 2008, Oliveira et al., 2008, Hsieh et al., 2010, Oliveira et al., 2011). This covalent bond is most likely formed after aerobic purification due to the oxidation of a π-cation radical species at the siroheme. This ability to produce π-cation radical species permits the generation of an extra electron at the active site, which may be one of the reasons for which sirohemes have been selected for the reduction of sulfite (Crane & Getzoff, 1996, Oliveira et al., 2008). Moreover, it was also shown by mass spectrometry that in solution the DsrAB from *D. norvegicum*, is present in several forms, having no (α_2β_2), one (α_2β_2γ) or two (α_2β_2γ_2) DsrC proteins coupled to

![Figure 1.10](image-url)
DsrAB (Oliveira et al., 2011). Oliveira et al proposed a mechanism for sulfite reduction in which they suggest that DsrAB would reduce sulfite to an $S^0$ valence state that would bind to the Cys$_A$ of the C-terminal arm of DsrC resulting in a persulfide (Oliveira et al., 2008). This DsrC persulfide would then suffer an internal reaction with Cys$_B$ with the release of hydrogen sulfide ($H_2S$) and with the formation of a disulfide bond between Cys$_A$ and Cys$_B$. This disulfide would then react with DsrK (from the DsrMKJOP membrane complex), becoming reduced to enter in another cycle of sulfite reduction (Figure 1.11) (Oliveira et al., 2008).

![Figure 1.11. Schematic representation of the proposed sulfate reduction mechanism. Sat, sulfate adenylyltransferase; ApsAB, adenosine phosphosulfate reductase; QmoABC, membrane complex that is the probable electron donor to ApsAB. C-SH represents the thiol group of Cys 93, C-S-SH a persulfide group of Cys 104, and C-S-S-C the disulfide bond between the two Cys. (From Oliveira et al. 2008)](image)

This proposal brought a new perspective to sulfite reduction since it linked this reaction to energy conservation through the DsrMKJOP complex. According to Oliveira et al. (2008) two electrons, from the total
of six electrons needed for the reduction of sulfite to sulfide, would come from the DsrC reduced terminal cysteines. These cysteines are converted to a disulfide bond state after reacting with the $S^0$ valence state and releasing $H_2S$. This DsrC disulfide form would then be reduced by DsrMKJOP membrane complex from which two electrons from the menaquinone pool would be needed. This proposal rejects the trithionate pathway and suggests that the subproduct formation of trithionate and thiosulfate were only \textit{in vitro} products caused by the absence of DsrC in the cavity of DsrAB and the excess of sulfite (Oliveira \textit{et al.}, 2008, Parey \textit{et al.}, 2010).

In the sulfur oxidation pathway it was shown that a mutant with a deletion in the \textit{dsrC} gene was genetically unstable and unable to grow even in the absence of sulfur compounds (Cort \textit{et al.}, 2008). Moreover, it was shown by Stockdreher and coworkers that, in \textit{in vitro} assays, DsrC is extremely important for sulfur oxidation as it receives sulfur from the DsrE protein (Stockdreher \textit{et al.}, 2012). In more detail, during

![Figure 1.12. Model of sulfur oxidation in A. vinosum integrating a sulfur transfer function for DsrEFH and a substrate donating function for DsrC (Stockdreher \textit{et al.}, 2012).](image-url)
oxidation, sulfur is first accumulated in sulfur globules, and then imported into the cytoplasm were it reacts with Cys\textsubscript{78} of DsrE (that belongs to DsrEFH cytoplasmic complex) (Stockdreher \textit{et al.}, 2012). This sulfur in DsrE is then proposed to be transferred to the Cys\textsubscript{A} of DsrC forming a persulfide which transfers it to the reverse DsrAB forming an intramolecular disulfide bond between Cys\textsubscript{B} and Cys\textsubscript{A}. This process is proposed to finalize in the DsrMKJOP complex where the oxidized disulfide DsrC form can be reduced, ready to enter in another cycle of sulfur relay (Figure 1.12) (Stockdreher \textit{et al.}, 2012).

### 1.5 How is dissimilatory sulfate reduction linked to energy conservation?

All SRP share a group of proteins known to be directly involved in sulfate reduction which include, Sat, ApsBA and DsrAB. However, these are cytoplasmic proteins and cannot be directly involved in the generation of a proton-motive force. To accomplish energy conservation the terminal reductases (ApsBA and DsrAB) must be linked to a membrane complex. There are several membrane complexes present in SRP that can be linked to energy conservation such as QmoABC, DsrMKJOP, Qrc and the Hmc/Tmc/Nhc family (Pereira \textit{et al.}, 2011). However, according to Pereira \textit{et al.} only two membrane complexes are conserved in all SRP and also present in many SOB (Pereira, 2008, Frigaard & Dahl, 2009, Pereira \textit{et al.}, 2011). These are the “quinone-interacting membrane-bound oxidoreductase” (QmoABC) and the dissimilatory sulfite reductase membrane complex (DsrMKJOP). These two complexes are thought to be implicated in energy conservation of SRP and have subunits that are homologous to subunits of heterodisulfide reductases (Hdr) of methanogens, complex responsible for the last step of methanogenesis: the reduction of heterodisulfide of
two thiol coenzymes (CoMSH and CoBSH) formed upon the release of methane (Thauer et al., 2008).

The Qmo membrane complex is generally composed of three subunits QmoA and QmoB (cytoplasmic subunits) and QmoC (membrane subunit), and is usually found in a sat-aprBA-qmoABC gene cluster (Meyer & Kuever, 2007, Pereira et al., 2011). However, in many clostridial SRP the qmoC gene is absent (Pereira, 2008, Pereira et al., 2011), and is proposed to be substituted by the hdrBC genes found in the vicinity of the qmoAB genes, suggesting a possible interaction (Pereira et al., 2011). The Qmo complex contains two FAD groups, two hemes b and several iron-sulfur centers. The two hemes b present in QmoC can be reduced by quinols. In 2010, Zane and coworkers verified that a D. vulgaris Hildenborough qmoABC mutant was not able to grow on sulfate but it grew on sulfite or thiosulfate (Zane et al., 2010). This confirmed that the QmoABC complex is essential for the conversion of APS to sulfite in the dissimilatory sulfate reduction mechanism. It was recently shown by Kaster and coworkers that, in methanogens, HdrABC forms a complex with MvhADG (methyl-viologen-reducing [NiFe]-hydrogenase) coupling the favorable H₂ reduction of CoM-S-S-CoB to the unfavorable reduction of ferredoxin (Kaster et al., 2011). They named this mechanism flavin-based bifurcation. Taking into account the homology between Qmo/Hdr and based on the bifurcating mechanism, Ramos et al. recently proposed a confurcation mechanism where QmoABC couples the oxidation of menaquinol (by QmoC) and the oxidation of a cytoplasmic reductant with low redox potential (via QmoB),
both acting as electrons donors to Qmo, to confurcate electrons to reduce ApsAB through QmoA (Figure 1.13) (Ramos et al., 2012).

The other membrane complex present in SRP and SOB, which proved to be essential for sulfur oxidation (Pott & Dahl, 1998), is DsrMKJOP. This complex was first identified in Allochromatium vinosum, a purple SOB, as part of the dsr locus responsible for the intracellular oxidation of sulfur (Pott & Dahl, 1998). The Dsr complex was first purified and characterized from the archaeon A. fulgidus, where it was named Hme for Hdr-like menaquinol-oxidizing enzyme (Mander et al., 2002). It was also shown that this complex is essential for sulfur globule oxidation in SOB (Sander et al., 2006). DsrMKJOP is a transmembrane complex with two subunits in the periplasm (DsrJ and DsrO), two in the membrane (DsrM and DsrP) and one cytoplasmic (DsrK). According to the subunit organization this complex can be divided in two modules: DsrMK (strictly conserved among SRP) and the DsrJOP (absent in some SRP, mainly Firmicutes) (Pereira et al., 2011, Grein et al., 2013, Rabus et al., 2015). The DsrJOP module is predicted to be involved in electron transfer.
between the periplasm and the menaquinone pool. The DsrJ protein contains three heme c-type binding motif (CXXCH), proposed to have three different distal axial ligands: His/His, His/Met and His/Cys. A His/Cys distal axial ligation is very unusual and has only been found in three other proteins, TsdA (Denkmann et al., 2012), PufC (Alric et al., 2004) and SoxXA (Reijerse et al., 2007). Grein and coworkers observed that the A. vinosum mutant lacking DsrJ was unable to oxidize intracellular sulfur, indicating that DsrJ is crucial for sulfur oxidation. Also, when this mutant was complemented with the dsrJ gene from D. vulgaris, it was able to fully restore the phenotype of the wild type, even though these genes only share 34% of identity, indicating that DsrJ might perform the same function in both organisms with opposite sulfur mechanisms (Grein et al., 2010). The individual midpoint redox potentials of DsrJ from A. vinosum are -20, -200 and -220 mV (Grein et al., 2010). These values are close to the calculated values for the His/Cys-ligated heme of PufC -160 mV (Alric et al., 2004), but far from -432 mV value reported for the SoxXA heme (Reijerse et al., 2007). Pires et al also reported that the His/Cys-ligated heme present in DsrMKJOP complex from D. desulfuricans was not fully reduced even at -400 mV (Pires et al., 2006). Overall, the DsrJ protein still remains an enigmatic protein with no clear attributed function. DsrO contains, by sequence analysis, four iron-sulfur clusters (three in the case of Desulfovibrio spp (Pires et al., 2006)) and a Tat signal peptide for translocation across de membrane. DsrP is an integral membrane protein with ten predicted transmembrane helixes, probably responsible for menaquinone/menaquinol interaction, as it belongs to the quinone-interacting NrfD/PsrC protein family (Grein et al., 2010). The DsrMK module is homologous to the membrane-boundHdrED. The HdrED complex is present in methylotrophic methanogens, and it is responsible
for the reduction of CoM-S-S-CoB to CoM-SH and CoB-SH (Figure 1.14) (Deppenmeier, 2004). An isolated DsrMK complex has been isolated from *Archaeoglobus profundus* (Mander *et al.*, 2004). DsrM is an integral membrane protein, but smaller than DsrP, with two *b*-type hemes and only five predicted transmembrane helices. It shares similarity with heme *b*-containing NarI of bacterial nitrite reductases and HdrE of Hdr from methanogenic archaea, which suggests an ability to oxidize the menaquinol pool (Grein *et al.*, 2010). Also, it was shown that the DsrM hemes *b* can be reduced with menaquinol analogues (Pires *et al.*, 2006). Finally in the cytoplasm resides DsrK. The homology of DsrK to the HdrD protein immediately argues that it should be a catalytic subunit of DsrMKJOP complex. The HdrD subunit is responsible for reducing the CoM–S–S–CoB (Figure 1.14) (Hedderich *et al.*, 2005). DsrK and HdrD share a CCG domain that contains the conserved sequence CX₉CCGX₉CXXC that binds the [4Fe-4S] center responsible for heterodisulfide reduction. Moreover, DsrK has no transmembrane helix but was suggested to be membrane anchored by Grein and coworkers, through an amphipathic α-helix at the N-terminus (Grein *et al.*, 2010), because in *A. vinosum* purifications DsrK was only found in the

---

*Figure 1.14. Scheme of the function of the HdrED complex from methylotrophic methanogens* (Kulkarni *et al.*, 2009).
membrane fractions. As already referred in chapter 1.4, DsrK has the
ability to interact with DsrC and due to this it is considered the link
between the reduction of sulfite by DsrAB and energy conservation in
SRP (Figure 1.15) (Pires et al., 2006, Oliveira et al., 2008, Pereira et al.,
2011).

Figure. 1. 15. The reduction of sulfite by DsrAB generates a S0 intermediate that reacts
with CysA of DsrC resulting in a persulfide, which by displacement of sulfide forms an
intramolecular disulfide bridge, DsrCox. This oxidized form of DsrC is reduced by the
DsrK protein of the DsrMKJOP complex (Grein et al., 2013)
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Chapter 1


Introduction


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Chapter 1


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Chapter 2

Dissimilatory sulfite reductase and DsrC: isolation and analytic assays
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Chapter 2

2.1. Introduction

As referred in the previous chapter, there has been a lot of debate concerning the last step of dissimilatory sulfate reduction, the six electron reduction of sulfite to sulfite. There are two mechanisms proposed: i) the trithionate pathway, in which the DsrAB reduces sulfite to trithionate, thiosulfate and sulfide, and ii) the DsrAB/DsrC pathway, in which DsrC cooperates with DsrAB in the reduction of sulfite. This last mechanism was proposed by Oliveira et al. (Oliveira et al., 2008), and implies the reduction of sulfite to an $S^0$ intermediate by DsrAB, which will interact with the Cys$_A$ conserved cysteine of DsrC to release sulfide.

In this chapter we will describe the preparatory work required to set up the experiments described in Chapter 3, whose aim was to elucidate the mechanism of sulfite reduction by DsrAB and the role played by DsrC. For this two DsrABs were isolated, one from *D. vulgaris* and the other from *A. fulgidus*, and several recombinant DsrC mutants from *A. fulgidus* were prepared. The two DsrABs enzymes are structurally similar and evolutionarily related (Müller et al., 2015) presenting the same quaternary structure, with only two main differences: i) the DsrAB from *D. vulgaris* has two non-catalytic sirohydrochlorines per each DsrA subunit instead of the two, also non-catalytic, sirohemes present in the DsrAs from *A. fulgidus* and ii) the DsrAB from *D. vulgaris* is always purified as a complex with none, one or two DsrC proteins ($\alpha_2\beta_2$ (minor), $\alpha_2\beta_2\gamma$ and $\alpha_2\beta_2\gamma_2$), while in the case of DsrAB from *A. fulgidus* the protein is purified without DsrC ($\alpha_2\beta_2$) (Oliveira et al., 2008, Schiffer et al., 2008, Oliveira et al., 2011). Due to the fact that DsrAB from *D. vulgaris* cannot be purified without DsrC, the DsrAB from *A. fulgidus* was used to test the role of DsrC in the proposed mechanism by Oliveira et al. (Oliveira et al., 2008), using also recombinant DsrC protein from the same organism.
Moreover, to identify the role of each conserved cysteine two DsrC variants with mutations in these cysteines were generated.

### 2.2. Materials and Methods

**Purification of DsrABs**

To study the dissimilatory reduction of sulfite to sulfide by the DsrAB, two dissimilatory sulfite reductases were purified: DsrAB from *D. vulgaris* str. Hildenborough (DSM 644) and *A. fulgidus* VC16 (4304).

**DsrAB from D. vulgaris.** *D. vulgaris* Hildenborough (DSM 644) cells were grown at 37 °C in a 300 L batch culture in a modified lactate/sulfate medium ([Oliveira et al., 2008](#)). The soluble cell fraction was obtained as previously described ([Oliveira et al., 2008](#)). All purification procedures were performed under aerobic atmosphere at 4 °C using an AKTA FPLC (Amersham Biotech Pharmacia) with two buffers, (A) 20 mM TrisHCl and (B) 50 mM TrisHCl with 1 M NaCl (both pH 7.6 and containing 10% glycerol). Buffer (A) was used to equilibrate the columns and buffer (B) to generate the ionic strength gradient. The soluble cell fraction was loaded in a Q-Sepharose fast-flow (XK50/30) column, and a stepwise salt gradient applied, with the DsrAB-containing fraction eluting at 300 mM NaCl. The characteristic *D. vulgaris* DsrAB absorption peak at 630 nm was used to track the protein, as previously described ([Wolfe et al., 1994](#), [Marritt & Hagen, 1996](#)). DsrAB-containing fractions were then loaded in a Q-Sepharose fast-flow (2.6 cm × 10.0 cm, Amersham Pharmacia Biotech) column and eluted in 250 mM NaCl. To verify enzyme purity, the final DsrAB-containing sample was analyzed by 12% SDS-PAGE gel electrophoresis. DsrC is present in the DsrAB
preparation from *D. vulgaris*, but remains functionally inactive during *in vitro* assays as previously described (Oliveira *et al.*, 2008).

**DsrAB from A. fulgidus.** *A. fulgidus* VC16 was kindly supplied by Doctor Luís Gafeira at Cell physiology and NMR laboratory coordinated by Professor Helena Santos at ITQB. *A. fulgidus* VC-16 (DSM 4304) was grown in sulfate/thiosulfate-lactate medium in a 300 L fermenter at 83 °C, as previously described (Stetter *et al.*, 1987), with some changes: the yeast extract was 0.05% (w/v), growth temperature was 83 °C, the medium was buffered with 20 mM PIPES sodium salt from sigma and 0.5 mM of sodium sulfide was used as initial reductant. Frozen cells were resuspended in 20 mM potassium phosphate (KPi) buffer pH 7 (buffer A), homogenized and disrupted in a French Press in the presence of DNase. The lysate was centrifuged at $58545 \times g$ for 20 min, followed by ultracentrifugation at $100000 \times g$ for 2 h. The supernatant was loaded in a Q-Sepharose high performance column (2.6 cm × 10.0 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. DsrAB was eluted at 0.4 M KCl using a stepwise gradient (0-1 M KCl). This fraction was concentrated and desalted by ultrafiltration (10 kDa cutoff, Amicon, Millipore) with subsequent dilution with buffer A. The desalted protein was applied to a Resource-Q column (1.6 cm × 3.0 cm, Amersham Pharmacia Biotech) and eluted at 0.25 M KCl using a stepwise gradient (0-1 M KCl). This fraction was concentrated and desalted by ultrafiltration with buffer A containing 5% (v/v) glycerol. This two-step purification protocol was performed according to (Schiffer *et al.*, 2008). Protein was quantified by the extinction coefficient at 593 nm of 60 mM$^{-1}$cm$^{-1}$ (Dahl *et al.*, 1993).
DsrAB activity assays

**Warburg activity assays.** The enzymatic activity of sulfite reduction was initially measured in a Warburg equipment with 50 mM KPi pH 7 at room temperature (with *D. vulgaris* DsrAB) using 1 mM of methyl viologen (Mv) reduced with the *Desulfovibrio gigas* [NiFe] hydrogenase (purified as described (Romão *et al.*, 1997)) as electron donor, in the presence of hydrogen, 430 nM of DsrAB and sodium sulfite (up to 1 mM). The Warburg flasks were equipped with manometers filled with Krebs fluid (428 mM NaBr + 0.465 mM Triton X-100 + 0.512 mM acid fuchsin). The enzymatic assays were stopped by the addition of 2 M of sulfuric acid. The Warburg flasks have two side arms and one main reservoir (where the reaction takes place). The buffer, Mv and hydrogenase are placed in the main reservoir, while the stopping reagent and the sulfite reductase were in each side arm. The flask was capped and the atmosphere was flushed with hydrogen until the Mv was completely reduced (intense blue), after which the system was closed and the initial value at the manometer was annotated. The reaction started by adding the enzyme in the side arm. The activity was measured by the decrease in hydrogen pressure inside the closed system. To calculate the amount (µL) of hydrogen consumed in the Warburg flask the following formulas were used:

\[
\mu L_{H_2} = h \times k \quad (1)
\]

\[
k = \frac{V_g \times \frac{273}{T} + V_f \times \alpha_{H_2}}{P_0} \quad (\mu L/mm) \quad (2)
\]

In equation (1), *h* is the value of the volume of the Krebs fluid in the manometer and *k* the constant calculated by equation (2), in which *V_g* is the gas volume of the empty flask and the *V_f* is the volume of the reaction. The \( \alpha_{H_2} \) at 23 °C = 0.0177 (solubility of hydrogen at 23 °C) an \( P_0 = 10000 \)
mm (Hg pressure expressed in terms of manometric fluid) were used for our experiments.

**Anaerobic chamber assays.** Another assay for enzymatic reduction of sulfite, thiosulfate and trithionate was performed inside an anaerobic chamber (95% Ar, 5% H₂) at 60 °C (*A. fulgidus*) or 37 °C (*D. vulgaris*) using reduced methyl viologen (Mv) as electron donor. The assays consisted of 50 mM KPi pH 7, 1 mM methyl viologen previously reduced with zinc granules, 430 nM of DsrAB, sodium sulfite (up to 0.5 mM) and, when added, recombinant DsrC (7.5 to 500 μM, WT or variants). The reaction started by addition of sulfite. Methyl viologen oxidation was monitored at 732 nm (ε=3.15 mM⁻¹.cm⁻¹) in a spectrophotometer (Shimadzu UV-1800). In all experiments where DsrC was used, it was previously reduced with 4 mM dithiothreitol (DTT) during 30 min at 37 °C, and the excess reductant was removed with a HiTrap Desalting column (GE Healthcare). Reduced DsrC was concentrated and kept under anaerobic conditions.

**Anaerobic vials experiments.** All solutions were prepared in an anaerobic chamber in previously boiled water, cooled under oxygen free N₂. *In vitro* reactions were carried out in 10 mL acid-washed, autoclave-sterilized, glass vials sealed with rubber septa. Each vial contained 50% buffer and 50% gaseous headspace. The reaction solution contained 50 mM KPi buffer (pH 7), varying sodium sulfite concentrations, 1 mM methyl viologen, 430 nM of DsrAB from *D. vulgaris* or *A. fulgidus*, and 8.25 nM [NiFe]-hydrogenase (297 U/mg). After removing the capped reaction vials from the chamber, the headspace was exchanged to 100% H₂ to initiate the experiments. Periodically samples were removed for sulfur compound analysis by HPLC.
Chapter 2

Cloning, expression and purification of A. fulgidus DsrC

The A. fulgidus VC16 dsrC gene (AF2228) was amplified by PCR using genomic DNA and primers (#1/#2 in Table 2.2) with EcoRI and HindIII restriction sites, respectively. The digested PCR product was cloned into pPR-IBA-2 (IBA, Germany), which allows insertion of a Strep-tag® II at the N-terminus, yielding plasmid IBAAFDsrC. The dsrC Strep-tag-encoding region was subcloned into the pET-22b(+) vector (Novagen) using Ndel and HindIII resulting in pET22AFDsrC. Site-directed mutagenesis was performed using the NZYMutagenesis kit (NZYTech) to replace two structural Cys (C77 and C85) for Ala, generating DsrCC77A/C85A (rDs2C) with #3/#4 primers. The DsrCC77A/C85A/C114A (CysAAla variant) and DsrCC77A/C85A/C103A (CysBAla variant) variants were constructed using the template vector for DsrCC77A/C85A. DsrC variants were amplified using #5/#6 primers for DsrCC77A/C85A/C114A, and #7/#8 primers for DsrCC77A/C85A/C103A. All cloned vectors were verified by sequencing.

The recombinant plasmids were transformed in E. coli BL21 Gold (DE3) cells (Stratagene) containing the pRARE2 vector (for codons rarely used in E. coli) and were grown at 37 °C in LB medium supplied with ampicillin (100 μg/mL) and chloramphenicol (30 μg/mL) until an OD₆₀₀ of 0.4. At this stage 500 μM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added and growth was continued for another 3 h. Cells were then collected by centrifugation at 6000 × g for 10 min and stored at -20 °C for short periods or -80 °C for long time preservation.

E. coli cells were resuspended in 100 mM TrisHCl pH 8 with 150 mM NaCl (buffer W) and disrupted in a French Press in the presence of
DNase and a protease inhibitor cocktail (Roche Diagnostics). The extract was centrifuged for 20 min at 58545 × g and the supernatant was subjected to a heat shock at 70 °C for 10 min followed by centrifugation for 5 min at 58545 × g to remove precipitated proteins. The supernatant was loaded into a Strep-Tactin affinity column (IBA) equilibrated with buffer W. DsrC was eluted with buffer W containing 2.5 mM desthiobiotin, and was concentrated and dialyzed to 50 mM potassium phosphate pH 7.
Table 2.1. List of strains and plasmids used in this study.

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<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics</th>
<th>Source or reference</th>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>E. coli α-Select</strong></td>
<td>F&lt;sup&gt;−&lt;/sup&gt; deoR endA1 relA1 gyrA96 hsdR17 (r&lt;sup&gt;K&lt;/sup&gt; m&lt;sup&gt;K&lt;/sup&gt;) supE44 thi-1 Δ(lacZYA-argFV169) φ80lacZΔM15 λ&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Bioline</td>
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<td><strong>E. coli BL21 Gold (DE3)</strong></td>
<td>F&lt;sup&gt;−&lt;/sup&gt; dcm&lt;sup&gt;+&lt;/sup&gt; The ompT hsdS (r&lt;sup&gt;B&lt;/sup&gt; m&lt;sup&gt;B&lt;/sup&gt;) gal&lt;sup&gt;+&lt;/sup&gt; (DE3) endA Tet&lt;sup&gt;R&lt;/sup&gt;</td>
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<td><strong>Plasmids</strong></td>
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<td>IBAAFDsrC</td>
<td>EcoR1 – HindIII fragment of PCR-amplified dsrC from A. fulgidus in pPR-IBA2; intermediate plasmid encoding DsrC with N-terminal Strep-Tag II; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Novagen</td>
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<td>This study</td>
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Table 2.2. List of primers used for plasmids construction.

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<tr>
<td>#2 DsrCAF-r</td>
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<tr>
<td>#3 DsrCC77AC85A-f</td>
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<td>CTTCCAAGCCAACGCGGTCTAAGCTTGGGCGG</td>
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<tr>
<td>#6 DsrCC77AC85AC114A-r</td>
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<tr>
<td>#8 DsrCC77AC85AC103A-r</td>
<td>CAATTCTAGCAGCTTTCGAGGTC</td>
</tr>
</tbody>
</table>

Determination of sulfur compounds

**Colorimetric detection methods.** To quantify sulfite colorimetrically we used the ‘Fuschin’ assay adapted from Grant (Grant, 1947). Standards of sodium sulfite (Na$_2$SO$_3$ anhydrous, analytical grade) were prepared immediately before the assay with deoxygenated water (boiled and degassed with N$_2$) or KPi buffer (prepared anaerobically). The reaction mixture is composed of 0.04% w/v Pararosaniline-HCl (analytical grade) in 10% H$_2$SO$_4$ (analytical grade) v/v, stored in an aluminum-foil wrapped tube or amber-glass bottle at 4°C; and 3.7% formaldehyde prepared fresh each day by diluting 37% (stock) formaldehyde 1:10 water. The reaction was performed on the bench working under N$_2$ flow, or in an anaerobic chamber. A detailed step-by-step protocol is available in (Leavitt, 2014).

Trithionate and thiosulfate were measured by a modified cyanolysis protocol (Kelly and Wood, 1994; Kelly et al., 1969; Sörbo, 1957). The
method was adapted from the method of Kelly and Wood (Kelly and Wood, 1994) in the following: the reaction volumes were reduced to 10 mL (in volumetric flasks) and with nitric acid as it was used in the original version of this method (Sörbo, 1957). Samples were added to the reaction buffer to fit within the range of ferric thiocyanate standards (prepared from potassium thiocyanate as a simple standard and thiosulfate as a reaction standard) from 5 to 25 µM (final concentration in the 10 mL reaction), as well as ‘blanks’ prepared from the in vitro assay reaction buffer (50 mM potassium phosphate buffer at pH 7). This was typically 400 µL of in vitro solution added to the 10 mL cyanolysis reaction, in duplicate per method (2X thiosulfate determinations and 2X trithionate determinations). A detailed step-by-step protocol is available in (Leavitt, 2014).

For sulfide quantifications, we preserved samples in zinc acetate (2% w/v) from each closed system reaction, using a modified Cline (Cline, 1969) method. Analytical grade sodium sulfide (>98.9% Na₂S·9H₂O) was used as the standard, and prepared in deoxygenated (boiled and N₂-sparged) in vitro reaction buffer, by precipitating the sulfide with excess zinc acetate (anhydrous), mimicking our sampling protocol. A detailed step-by-step protocol is available in (Leavitt, 2014).

**HPLC analysis.** Thiol compounds were analyzed by HPLC after derivatization with Monobromobimane (mBbr). mBbr (Life Technologies) was diluted in HPLC ultra-pure acetonitrile to a final concentration of 50 mM. For the derivatization protocol, described in (Fahey & Newton, 1987), 10 µL of sample from the enzymatic reaction were derivatized with two fold excess of mBbr versus the initial concentration of sulfite substrate, in 20 mM HEPES buffer pH 8, in a final volume of 86 µL. The reactions were performed in the dark during 10 min, after which 4 µL of methanosulfonic acid 5 M were added to stop the reaction.
Derivatized sulfur compounds (sulfite, thiosulfate and sulfide) were analyzed by HPLC (Waters Alliance 2695) equipped with a fluorescence detector (Waters 486) with an excitation wavelength of 380 nm and emission wavelength of 480 nm, using a reverse phase ultrasphere C18 column (4.6 mm × 25 cm, 5 μm, Beckman Coulter) at 35 °C. Derivatized samples were diluted with 10 mM methanosulfonic acid. The gradient was performed with 0.25% acetic acid pH 4 in MilliQ water (solvent A) and 100% methanol (solvent B), at 1.20 mL.min⁻¹. The elution protocol was the following (% of solvent B): 0 min, 20%; 5 min, 20%; 13 min, 50%; 16 min, 52%; 20 min, 100%; 24 min, 100%; 26 min 20% and 31 min, 20%. The retention times were: 3.44, 5.6 and 15.12 min for sulfite, thiosulfate and sulfide, respectively. Calibration curves were performed with sodium thiosulfate, sodium sulfite and sodium sulfide nonahydrate, using freshly prepared solutions at pH 7.

Trithionate does not react with mBbr and was determined according to Jeffrey and coworkers (Jeffrey & Brunt, 2007), also using HPLC (Waters Alliance 2695) equipped with a UV-visible detector (Waters 2487) at the wavelength of 192 nm using a Dionex IonPac AS16 RIFC column (4 mm × 25 cm) coupled to a Dionex IonPac AG16 RIFC pre-column (4 mm × 5 cm), at 35 °C. An isocratic gradient was performed with 150 mM of sodium perchlorate in MilliQ water at 1 mL.min⁻¹. The retention time of sodium trithionate is 5.6 min. The calibration curve was performed with sodium trithionate standard, kindly synthesized by A. Masterson from Professor David Johnston’s Laboratory at Harvard University, diluted in the enzymatic reaction mixture.
MalPEG gel-shift assays (GSA)

The MalPEG gel-shift assay was used to monitor the redox state of DsrC cysteines as described (Venceslau et al., 2013). Briefly, at different time points during the enzymatic sulfite reduction assay a sample of reaction mixture corresponding to 5 µg of DsrC were removed and immediately mixed with 1 mM MalPEG (methoxy-polyethylene glycol maleimide, MW 5000 g.mol\(^{-1}\), Fluka) for 15 min at 37 °C inside the anaerobic chamber. The reaction was terminated by adding an equal volume of 2xSDS loading buffer (38 mM TrisHCl buffer pH 6.8 with 10% glycerol, 6% SDS, 0.05% bromophenol blue) and the sample was analyzed by 10% Tricine-SDS-PAGE without boiling, under non-reducing conditions, followed by staining with Coomassie Blue.
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2.3. Results and discussion

Growth and purifications

There was no previous experience in the lab of growing *A. fulgidus*. Our first approach was to optimize the growth conditions of *A. fulgidus* VC16. When we started growing this organism the maximum optic density at 600 nm (OD<sub>600</sub>) after 24 h was ~0.300. After several attempts with more thiosulfate, sulfate and both, the growth of *A. fulgidus* was never higher than OD<sub>600</sub> 0.350. A significant improvement was achieved by the removal of sodium dithionite as a reducing agent and the increase of the PIPES buffer concentration from 8.23 mM to 20 mM, resulting in a maximum OD<sub>600</sub> after 24 h of ~0.500. With this protocol a 300 L fermenter was performed, with a yield of 0.38 g/L in a total of ~112 g of wet cells of *A. fulgidus*.

The main difference between the two DsrAB (*D. vulgaris* Vs *A. fulgidus*) is the presence/absence of DsrC. In a native gel the DsrAB from *D. vulgaris* always appears in two bands (corresponding to DsrAB bound to one (named Fast form) or two (named slow form) DsrC proteins) (Figure 2.1 A), whereas the DsrAB from *A. fulgidus* displays only one band (Figure 2.1 B) (Dahl *et al.*, 1993, Oliveira *et al.*, 2008). Both proteins were purified with a ratio 280/391 nm of 2.9 for DsrAB from *D. vulgaris* and 2.4 for DsrAB from *A. fulgidus*. 
Activity assays

**DsrAB from *D. vulgaris***. The activities for sulfite reduction by DsrAB from *D. vulgaris* were measured with two different methodologies: Warburg method and sulfite consumption analysis by HPLC.

Our first approach was the use of the Warburg equipment. This methodology measures the hydrogen pressure in a closed system. In this system *D. gigas* [NiFe] hydrogenase was used to reduce the Mv, which was used as the artificial electron donor for DsrAB to reduce sulfite. Through the consumption of the hydrogen by the hydrogenase we can indirectly measure the activity of the DsrAB.

In the conditions used for this assays, the Warburg method was shown to have low reproducibility for the analysis of sulfite reduction. A high variation between replicates was observed and could be attributed to several features: gas leaking from the Warburg flasks, differences in
bath temperature over time or differences in the activation of the hydrogenase. Due to these differences in the replicates, we decided to use a different method to evaluate the activity of sulfite reduction by DsrAB from *D. vulgaris*.

The second approach used was the determination of sulfite consumption. In these experiments closed anaerobic vials were used to harbor the reaction for sulfite reductase by DsrAB with Mv as electron donor reduced with Hase, under a H₂ atmosphere. Due to the slow activity of DsrAB alone its kinetic performance of sulfite reduction was analyzed after two hours of activity. At this time 10 µL sample was removed and analyzed by HPLC (Figure 2.2).

![Figure 2.2. Activity assays with *D. vulgaris* DsrAB at 37 °C. One unit (U) is defined as the quantity of enzyme that catalyzes the conversion of one µmol of substrate per minute.](image)

With this assay a $K_m$ of 4.1 mM and a $V_{max}$ of 238 mU/mg was determined for *D. vulgaris* DsrAB. This experiment also showed that there was no substrate inhibition of the activity of sulfite reduction by DsrAB of *D. vulgaris* at initial concentrations of sulfite as high as 50 mM.
**DsrAB from A. fulgidus.** The activity measurements for sulfite reduction by DsrAB of *A. fulgidus* were performed in the anaerobic chamber with the UV-visible spectrophotometer and with zinc reduced methyl viologen as the artificial electron donor. These assays were performed at 60 °C, even though the optimal temperature for sulfite reduction of this DsrAB is 80 °C. We did not perform the activity assays at the optimal temperature to avoid overheating of the chamber. To test the activity of DsrAB and to compare it to DsrAB of *D. vulgaris* the Kpi buffer was prepared at pH 7 instead of the optimal pH 6 (Parey *et al.*, 2010).

![Figure 2.3](image)

*Figure. 2.3. Activity assays with A. fulgidus DsrAB at 60 °C. One unit (U) is defined as the quantity of enzyme that catalyzes the conversion of one µmol of substrate per minute.*

These experiments (Figure 2.3) with DsrAB from *A. fulgidus* showed a $K_m$ of 13 µM and a $V_{max}$ of 46 mU/mg (similar to the value reported in (Parey *et al.*, 2010)). We also tested the activity with different substrates (sulfite, thiosulfate and thionitrate) with DsrAB in the same conditions (Figure 2.4). In the conditions in which we performed these assays...
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(pH 7), the same catalytic activity was observed for all three substrates. These results did not agree with the previous work of Parey and coworkers (Parey et al., 2010) that verified that at pH 7 the DsrAB from A. fulgidus had a higher activity with thiosulfate than with sulfite or trithionate.

Recombinant DsrC from A. fulgidus. DsrC has been an intriguing protein since it was first discovered, associated with DsrAB (Pierik et al., 1992). In 2008, the crystal structure of DsrAB from D. vulgaris Hildenborough showed how DsrC interacts with DsrAB. Oliveira and coworkers proposed that DsrC is a partner of DsrAB required for sulfite reduction (Oliveira et al., 2008).

To study the role of DsrC in DsrAB activity (discussed in chapter 3) we produced heterologous recombinant DsrC in E. coli. First the dsrC gene from A. fulgidus was cloned and inserted in pPR-IBA2 vector that inserts a strep-tag II in the N-terminal of the rDsrC, producing the vector IBAAFDsrC. The N-terminal was selected to avoid disturbing the strictly conserved C-terminal that interacts with DsrAB. This vector was transformed in E. coli BL21 (DE3) Gold with the pRARE2 vector to

Figure. 2.4. Activity measured for sulfite/thiosulfate/trithionate (500 µM) with DsrAB and methyl viologen as artificial electron donor.
produce the rDsrC protein. However, even after several growth optimizations (temperature, oxygen, and IPTG concentration) we never obtained yields of the rDsrC protein higher than 1 mg/L of culture. As so, a different approach was used, here we removed the dsrC gene along with the Strep-tag II from the IBAAFDsrC vector and cloned it in pET22b(+), without the 6-His tag present in this original vector. With this change the pET22-AFDsrC was generated. This vector was transformed in *E. coli* BL21 (DE3) Gold with the pRARE2 vector. As the DsrC protein belongs to an extremophile that grows at 83 °C we used temperature heat shock (70 °C) as the first purification step. This changes led to a 10 fold increase in the rDsrC protein yield.

The analysis of the interaction of DsrAB and rDsrC in the presence of sulfite and methyl viologen (Mv) as the artificial electron donor confirm what has suggested by Oliveira and coworkers, DsrC is indeed a partner of DsrAB increasing the sulfite activity by DsrAB at 60 °C (see chapter 3).

For analysis of the redox state of DsrC upon reaction with DsrAB we used the gel shift assay (GSA), set up by Venceslau et al. (Venceslau 2014). This assay uses the thiol-binding reagent MalPEG to induce a shift in the molecular mass of the protein that corresponds to the number of cysteines labelled.

With the GSA we realized that the two structural cysteines of DsrC would also bind the reagent (MalPEG). To simplify the GSA results we decided to generate a new rDsrC, in which the two structural cysteines (Cys 77 and Cys 85) were substituted by alanines. This new vector, pET22-AFDsrCC77A/C85A, was generated using the pET22-AFDsrC along with the primers #3 and #4. This vector was then transformed in *E. coli* BL21 (DE3) Gold with the pRARE2 vector, which led to the
production of the rDsrC2 protein. The final yield of 15 mg/L of culture was in the same range of rDsrC.

The two cysteines (Cys 77 and Cys 85) were considered structural residues for the stabilization of the protein at high temperature (Mander et al., 2005) (Figure 2.5). For this reason, we decided to do measure the stability of the new rDsrC2 variant, by comparing the temperature melting curve of the two proteins, rDsrC and rDsrC2, by Circular Dichroism. According to this experiment both proteins behaved equally showing that the absence of these cysteines in rDsrC2 did not reduce its stability to high temperature. Moreover, the kinetic behavior of rDsrC2 with DsrAB was similar to that of rDsrC.

Also to test the importance of DsrC in DsrAB sulfite reduction it is essential to understand the role of each cysteine. For this, two variants were generated from the pET22-AFDsrCC77A/C85A: the CysAAla

Figure. 2.5. Schematic representation of the DsrC from A. fulgidus. The red circle highlights the two cysteines (77 and 85) bounded by a disulfide bridge (yellow). The Pymol program was used to generate the cartoon (pdb 2A5W)
(mutation on the Cys 114) variant was generated using the primers #5 and #6, the CysBAla (mutation on the Cys 103) variant was generated using the primers #7 and #8. These two variants were also transformed in *E. coli* BL21 (DE3) Gold with the pRARE2 vector and being the total yield similar to rDsrC2.

**Substrate and products detection.** We first followed substrate (sulfite) consumption and detection of products using colorimetric methods such as Fuchsin (sulfite), Cyanolysis III and II (Thiosulfate and trithionate, respectively) and Cline (sulfide). These methods proved to be time consuming (for a large set of samples) and sample demanding (which required a large volume of enzymatic reaction). As so, HPLC measurement seemed a better option. To do so we optimized two HPLC methods to quantify the products that could be formed during DsrAB sulfite reduction: 1) the derivatization of the free thiols with monobromobimane (mBbr) for sulfite, thiosulfate and sulfide, and 2) a method for the detection of trithionate (without derivatization).
The mBbr method was based on the Fahey method (Fahey & Newton, 1987). We were able to reduce 49 min in the total run, and still accurately detect sulfite, thiosulfate and sulfide (Figure 2.6). This was achieved by increasing the temperature to 35 °C and the pH 4.

*Figure. 2.6. Overlapped HPLC chromatograms of the standards: sulfite (A), thiosulfate (B) and sulfide (C).*
The trithionate detection method had to be implemented in our laboratory. The problem behind the detection of trithionate by HPLC using the UV-visible detector was the absorbance of the mobile phase in the 190-200 nm region (Miura & Kawaoi, 2000). The use of sodium perchlorate as a mobile phase and an anion exchange column solved this problem (Jeffrey & Brunt, 2007). According to the trithionate UV-visible spectrum, the best absorbance region to detect this compound is at 192 nm. This method proved to be very good for the quantification of trithionate (Figure 2.7).

Figure 2.7. HPLC overlapped chromatograms of the standard trithionate (A).
2.4. Conclusions

This preparatory work was necessary to allow the analysis of sulfite reduction by DsrAB in the presence of its physiological partner, DsrC, described in the next chapter. We were able to purify DsrAB from *A. fulgidus* without the presence of the DsrC protein, contrary to what happens when purifying DsrAB from *D. vulgaris*. Also, the generation of the three recombinant DsrC variants (rDsrC2, Cys_{B}Ala and Cys_{A}Ala) enabled the understanding of the role of each cysteine for the reduction of sulfite by DsrAB (see chapter 3). The implementation of the two HPLC methods contributed for a fast analysis of the substrate and the products as well as to low the detection limit for all the chemical compounds analyzed.
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Chapter 3

A DsrC trisulfide links dissimilatory sulfate reduction to energy conservation
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One Sentence Summary: The physiological product of sulfite reduction, which governs the global sulfur cycle, is an intramolecular trisulfide in the protein DsrC, the reduction of which is coupled to energy conservation.

Abstract

Dissimilatory sulfate reduction is a microbially mediated process that has governed the Earth's biogeochemical sulfur cycle for the last 2 billion years. Marine sulfate is the Earth's largest mobile sulfur reservoir, representing a global sink for electrons. Over geological timescales, microbial sulfate reduction mediates the Earth's net oxidation state. In biogeochemistry, it has been used for decades as a proxy for atmospheric oxygenation, due to its strong signature in sulfur isotope discrimination. Yet the enzymatic mechanisms that generate the isotope effects in sulfate reduction are unknown. The key step in microbial sulfate reduction is the reduction of adenosine 5'-phosphosulfate to sulfite by ApsAB. Its biochemistry has been elusive. Here we show that the product of sulfite reduction by DsrAB is a Cys-S-S-S-Cys trisulfide between two conserved cysteines of the DsrC protein, which we show to be a physiological reaction substrate. Dissimilatory sulfate reduction couples the 4-electron reduction of the DsrC trisulfide to energy conservation.
Chapter 3

3.1. Introduction

Sulfur is a versatile element with extremely rich chemistry, and the breadth of its biological forms and mechanisms of action is still being unraveled. On a global scale the sulfur biogeochemical cycle is driven by microbial processes, most importantly marine sulfate (SO$_4^{2-}$) reduction. Sulfate reduction impacts the redox balance of the planet, over geological timescales, due to the burial of reduced sulfides. The pool of marine sulfate corresponds to an oxidant capacity 10-fold larger than that of atmospheric oxygen (Hayes & Waldbauer, 2006). Microbial sulfate reduction has a strong signature in sulfur isotope discrimination, used to trace sulfur cycling and the Earth’s redox environment in both ancient and modern settings (Berner & Canfield, 1989, Canfield, 1998, Farquhar et al., 2010, Halevy et al., 2012). This microbial process has a dissimilatory (respiratory) nature, and is performed by a specific group of organisms (sulfate reducing bacteria and archaea), in contrast to assimilatory sulfate reduction performed by many prokaryotic and eukaryotic organisms.

Both assimilatory and dissimilatory pathways of sulfate reduction (Figure 3.1) involve activation to adenosine 5’-phosphosulfate (APS) and subsequent reduction to sulfite (SO$_3^{2-}$). The sulfite reductases, responsible for the reduction of sulfite to sulfide in both pathways, have a siroheme coupled to a [4Fe-4S] cluster as catalytic cofactor (Crane & Getzoff, 1996, Simon & Kroneck, 2013). The assimilatory sulfite reductases (aSiRs) are NADH- or ferredoxin-dependent enzymes present in plants, fungi, bacteria and archaea, where they are responsible for the direct reduction of sulfite to sulfide (S$^{2-}$). This is further converted to cysteine, the precursor of other sulfur-containing...
biomolecules. The dissimilatory sulfite reductases (dSiRs) are present in sulfate reducers (Grein et al., 2013), where dSiR operates to reduce sulfite, and in other microorganisms that use sulfur compounds for their energy metabolism. These include phototrophic and chemotrophic sulfur oxidizing bacteria where dSiR presumably operates in reverse (Frigaard & Dahl, 2009), and many microorganisms that can reduce sulfite, thiosulfate (S$_2$O$_3^{2-}$) and organosulfonates (R-SO$_3$) or disproportionate sulfur compounds (Simon & Kroneck, 2013, Müller et al., 2015).

The most widespread dSiR is DsrAB, a key enzyme in prokaryotic sulfur metabolism (Grein et al., 2013). DsrAB is closely related to aSiR

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**Figure. 3.1. Comparison of the dissimilatory and assimilatory sulfate reduction pathways.** (A) Dissimilatory pathway used by sulfate reducing bacteria to respire sulfate, with production of sulfide. (B) Assimilatory pathway as operational in E. coli, producing cysteine, the precursor of other sulfur-containing biomolecules. In many bacteria and in plants, sulfite is also generated by direct reduction of APS.
and both proteins diverged from a common ancestor that was present in ancient organisms preceding the separation of the Archaea and Bacteria domains (Crane et al., 1995, Wagner et al., 1998). An unrelated heme c-containing dSiR was recently described (Hermann et al., 2015), but is present in only a few proteobacteria. Through its operation in sulfate reducers, DsrAB is believed to be a major player in determining sulfur isotope fractionations preserved in geological records and used to reconstruct the redox history of the Earth’s surface (Bradley et al., 2011).

Despite their close phylogenetic and structural relationship, a few fundamental differences distinguish DsrAB from aSiR. The latter carries out the direct six-electron reduction of sulfite to sulfide, whereas in vitro DsrAB produces a mixture of products ranging from trithionate and thiosulfate to fully reduced sulfide (Lee & Peck, 1971). This observation forms the basis for the long unresolved issue of whether dissimilatory sulfate reduction involves a direct reduction of sulfite to sulfide by DsrAB or whether trithionate and thiosulfate are necessary intermediates (Figure 3.2) (Peck et al., 1982, Brunner & Bernasconi, 2005).

![Figure 3.2](image_url)

**Figure. 3.2.** The two previously proposed pathways for dissimilatory sulfate reduction. The direct pathway involves the six-electron reduction of sulfite to sulfide by DsrAB. The trithionate pathway invokes the necessary production of trithionate and thiosulfate as intermediates in sequential two-electron reductions, and implies the actions of thiosulfate and trithionate reductases.
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This is a key question that shapes both biochemical and geochemical models of S cycling (Brunner & Bernasconi, 2005, Bradley et al., 2011). Another important difference between DsrAB and aSiR is that while aSiR processes small quantities of sulfite for assimilation, DsrAB must turn over large sulfite pools for respiration. In this light, it is surprising that the in vitro catalytic activity of DsrAB is much lower than that of aSiRs (Crane & Getzoff, 1996), hinting at a need for activating proteins. Finally, dissimilatory sulfite reduction must somehow be coupled to chemiosmotic energy transduction in order to conserve the free energy resulting from reduction of sulfite to sulfide ($\Delta G^\circ = -172$ kJ/mol for $\text{HSO}_3^-/\text{HS}^- \text{with H}_2$ as electron donor). Since DsrAB is a soluble protein not associated to the membrane (like aSiR), how energy is conserved during sulfite reduction is a critically important, yet unresolved question.

The crystal structure of DsrAB from the sulfate reducing bacterium Desulfovibrio vulgaris provided crucial insight into the process of sulfite reduction by showing the close and likely functional association to DsrC (Oliveira et al., 2008). DsrC is a small protein with a highly conserved C-terminal arm containing two strictly conserved cysteines: the penultimate residue, which we name CysA, and another found eleven residues before, which we name CysB (Figure 3.3) (Venceslau et al., 2014).
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Figure. 3.3. **Alignment of the DsrC C-terminal arm.** DsrC from sulfate reducing organisms (1), organisms that reduce sulfite/thiosulfate (2) and sulfur oxidizing bacteria (3) are included. Strictly conserved residues are in black. The key DsrC Cys_A and Cys_B are highlighted in red.

In the crystal structure of the *D. vulgaris* DsrAB-DsrC complex, this C-terminal arm inserts into a channel present between DsrA and DsrB such that Cys_A is positioned directly next to the substrate-binding site, suggesting a role in catalysis (Oliveira et al., 2008). A central role of DsrC in dissimilatory sulfite reduction is further supported by the observation that all genomes containing the *dsrAB* genes also encode *dsrC* (Venceslau et al., 2014), with the latter being one of the most highly expressed energy metabolism genes. This is true in both isolated organisms (Haveman et al., 2003, Keller et al., 2014) and in metatranscriptomic studies (Canfield et al., 2010, Stewart et al., 2011). DsrC is also part of a larger family of proteins that include TusE, a sulfur trafficking protein known to perform thiomodification of tRNAs via a persulfide relay system (Ikeuchi et al., 2006), and RspA, thought to have a regulatory role (Venceslau et al., 2014). While the two C-terminal cysteines in DsrC are always conserved, only Cys_A is conserved in TusE and neither Cys is in fact conserved in RspA (Venceslau et al., 2014).
3.2. Materials and Methods

Purification of *A. fulgidus* DsrAB.

The purification protocol is detailed in chapter 2.

Cloning, expression and purification of *A. fulgidus* DsrC

The cloning, expression and purification protocols for the recombinant DsrC from *A. fulgidus* are detailed in chapter 2.

DsrAB activity assays

The protocol for the sulfite activities of DsrAB and rDsrC are detailed in chapter 2. To assess the importance of DsrC Cys on the enzymatic activity of DsrAB, the two cysteines were either alkylated or oxidized to form a disulfide bond. Alkylation was performed by incubating DsrC with 50 mM iodoacetamide (Sigma) in 50 mM ammonium bicarbonate pH 7.8 during 30 min at 55 °C. The intramolecular oxidation of DsrC Cys was obtained by incubation with 1 M L-arginine (Sigma) for 2 h at 30 °C, as standard oxidizing conditions lead to formation of the dimer (Venceslau *et al.*, 2013). Excess of iodoacetamide or arginine was removed by centrifugal gel filtration (Micro Bio-Spin 6, Bio-Rad).

Determination of sulfur compounds by HPLC

The determination of the sulfur compounds by HPLC are detailed in chapter 2.
MalPEG gel-shift assays

The MalPEG gel-shift assays are detailed in chapter 2.

Determination of DsrC thiol content by DTNB

DsrC was subject to DTNB analysis to quantify the number of sulfhydryl groups present before and after the enzymatic assay with DsrAB. A sample of reduced DsrC (0.8 – 3 nmol) used for the enzymatic assay, was incubated in 100 mM potassium phosphate pH 8, 1 mM EDTA, and 200 µM DTNB (Sigma) for 15 min at room temperature. The production of 2-nitro-5-thiobenzoate anion (TNB²⁻) was quantified by measuring the absorbance at 412 nm using the extinction coefficient of 14150 M⁻¹ cm⁻¹ (Riddles et al., 1983). To measure the number of DsrC sulfhydryl groups after the enzymatic reaction with DsrAB and sulfite, this was extracted from the reaction mixture using a Strep-Tactin affinity column. After protein concentration, the same protocol as described above was performed.

Mass spectrometry

DsrC wt and variants were analyzed for intact mass and peptide mass fingerprinting by MALDI-TOF-TOF. The proteins were analyzed before addition to the sulfite reductase assay, i.e., in the reduced form, and at the end of the enzymatic assay.

**Intact mass.** The protein buffer was exchanged to 20 mM ammonium acetate pH 7.2 before analysis using a centrifugal gel filtration column (Micro Bio-Spin 6, Bio-Rad). To quantify the number of reduced cysteines, samples were incubated with 50 mM iodoacetamide
(Sigma) in 50mM ammonium bicarbonate pH 7.8 during 30 min at 55 °C still under anaerobic conditions. After DsrC alkylation the buffer was exchanged to 20 mM ammonium acetate pH 7.2. Samples (0.4 µL) were spotted directly onto the MALDI plate and were mixed in a 1:1 ratio with 10 mg/ml of matrix sinapinic acid dissolved in 50% (v/v) acetonitrile and 5% (v/v) formic acid, and allowed to air dry.

**Peptide mass fingerprinting.** Samples were first alkylated with iodoacetamide, as the described above, and run in a 10% Tricine-SDS-PAGE under non-reducing conditions. Gel slices containing DsrC were cut and digested with Asp-N (Roche, 20 ng/µl) overnight at 37 °C. This protease was chosen to generate a peptide containing both conserved C-terminal Cys: \(^{101}\text{DACRIAGLPKPTGCV}^{115}\), with 1500.8 Da. The digested peptides were desalted and concentrated using a POROS R2 column (Applied Biosystems) and eluted directly onto the MALDI plate using 0.6 µL of 5mg/ml alpha-cyano-4-hydroxycinnamic acid (Sigma) in 50% (v/v) acetonitrile and 5% (v/v) formic acid and air dried.

The data was acquired in linear MS mode using a 4800plus MALDI-TOF-TOF (AB Sciex) mass spectrometer and the 4000 Series Explorer Software v.3.5.5 (Applied Biosystems). External calibration was performed using Promix1 and Pepmix1 (Laser BioLabs) for intact mass and peptide mass fingerprinting techniques, respectively. The precursor ions of interest from the MS spectra were selected for MS/MS analysis. MS data was obtained by the Mass Spectrometry Unit (UniMS), ITQB/iBET, Oeiras, Portugal.
Generation of \textit{dsrC} complementation and deletion strains in \textit{D. vulgaris}

To try to generate a deletion mutant for the chromosomal \textit{dsrC} gene, plasmid pMO\text{Δ}dsrC was created to delete this gene by double homologous recombination in \textit{D. vulgaris} Hildenborough. This plasmid was obtained by sequence ligation independent cloning (SLIC; (Li & Elledge, 2007)) fusing four different fragments that were obtained by PCR amplification. The fragments corresponded to the upstream and downstream regions of the chromosomal \textit{dsrC} (primers \#9/ \#10 and \#11/ \#12), the kanamycin resistance gene and pUC origin/ Spec gene (primers \#13/ \#14 and \#15/ \#16) from pSC27 (Keller \textit{et al.}, 2011); were then added to the pMO719 background via SLIC. Products from the amplification were inserted into \textit{E. coli} α-select Silver Efficiency (Bioline®) and successful transformants were isolated on LB medium containing spectinomycin (100µg/ml) (Zane \textit{et al.}, 2010). Correct isolates were identified by the expected PCR amplicons from the plasmids constructs and also by sequencing. \textit{D. vulgaris} cells grown either on lactate-sulfate medium or on pyruvate medium (fermentative conditions) were electroporated with pMO\text{Δ}dsrC, according to (Zane \textit{et al.}, 2010, Keller \textit{et al.}, 2011). Despite numerous attempts, clones could never be obtained from the respective plates containing G418 (a kanamycin analogue to which the \textit{kan} gene also confers resistance (Zane \textit{et al.}, 2010)). Negative and positive controls were done simultaneously to exclude problems with the electroporation itself.

To overcome the inability to create a \text{Δ}dsrC deletion strain in \textit{D. vulgaris}, a second approach was followed to allow manipulation of the \textit{dsrC} gene by first introducing in the cell a plasmid for expression of \textit{dsrC},
then allowing the deletion of the chromosomal copy of *dsrC*, using pMO\(\Delta dsrC\). The pMO\(\text{PHisDsrC}\) plasmid was created for the production of His-tagged DsrC. Initially, *dsrC* was amplified from chromosomal DNA of *D. vulgaris* using primers #17 and #18 which fuse a 6xHis-tag to the N-terminus of the protein. The resulting fragment was digested with *NdeI* and *EcoRI* and ligated into pPR-IBA-2 (IBA, Germany) that has been digested with the same restriction enzymes. The *dsrC* His-tag fused fragment was subcloned into pMOIP5 (Ramos et al., 2012), giving rise to pMO\(\text{PHisDsrC}\) plasmid, whose expression is under control of the constitutive *aphII* promoter (Zane et al., 2010). The pMO\(\text{PHisDsrC}\) plasmid was inserted into *D. vulgaris* by electroporation, originating strain IPFG06. Subsequently, the pMO\(\Delta dsrC\) deletion plasmid was inserted into IPFG06 strain and transformants were selected on pyruvate plates containing geneticin and spectinomycin, resulting in IPFG07 strain. Two additional plasmids encoding DsrC with cysteine to alanine point mutations were created: pMO\(\text{PHisDsrCC26A/C93A}\) (Cys\(_B\)Ala variant; primers #19/ #20 and #21/ #22) and pMO\(\text{PHisDsrCC26A/C104A}\) (Cys\(_A\)Ala variant; primers #19/ #20 and #23/ #24), where Cys26 a non-conserved cysteine was mutated to Ala. Plasmids pMO\(\text{PHisDsrCC26A/C93A}\) and pMO\(\text{PHisDsrCC26A/C104A}\) were inserted into *D. vulgaris* resulting in IPFG08 and IPFG10 strains, respectively, both containing the chromosomal and plasmid copies of *dsrC*. Subsequently, the pMO\(\Delta dsrC\) deletion plasmid was inserted successfully into IPFG08 originating IPFG09 after selection. However, this procedure was not successful for the IPFG10 strain, despite multiple attempts. The pMO\(\text{PHisDsrC}\)-like plasmids were sequenced before and after insertion into *D. vulgaris*. The *dsrC* gene deletion in strains IPFG07 and IPFG09 versus WT and IPFG06 were confirmed by PCR using primers #25/#26 and Southern blot.
Growth experiments

*D. vulgaris* Hildenborough WT and variant strains (IPFG06, IPFG07 and IPFG09) were grown anaerobically at 37 °C in 100 mL flasks with 50ml of MOY medium (Zane et al., 2010) with lactate-sulfate (30-30 mM). All cultures were inoculated with 2% (v/v) freshly grown cells grown in pyruvate fermentation medium, which consists in MOY medium with pyruvate (60 mM) supplemented with a small amount of sulfate (2 mM). Antibiotics were added as follows: G418 at 400 µg/ml for IPFG07 and IPFG09, and spectinomycin at 100 µg/ml for all variants. For the growth curves only spectinomycin was maintained due to the presence of pMOIPHisDsrC-like plasmids. Growth was monitored by optical density (600 nm) with a spectrophotometer Shimadzu UV-1603. All optical density measurements are the mean of triplicates. The results for maximum OD and doubling times were statistically analyzed with SigmaStat 3.0, using the ANOVA method and pairwise multiple comparison procedures (Holm-Sidak test). The differences obtained between strains for both parameters were statistically significant (*p*-value ≤ 0.001).

For confirmation that the genotype was maintained, at the end of growth in lactate-sulfate medium cells were collected for DNA extraction (DNA Purification kit, Promega) and PCR was performed using primers #25/#26.
Western blot analysis of DsrC expression

Cells of *D. vulgaris* WT and variants grown in lactate-sulfate were collected at the end of exponential phase, and centrifuged for 12 min at 3000 × *g*. Cells were disrupted using the BugBuster® Protein Extraction Reagent (Novagen) for 20 min at room temperature and centrifuged at 16000 × *g* for 20 min at 4 °C. Crude extracts (40µg) were resolved on 10% Tricine-SDS-PAGE gels under reducing and denaturing conditions prior to transfer to PVDF membranes (transfer buffer: 48 mM Tris and 39 mM Glycine pH 9.2) using a Mini Trans-Blot® electrophoretic transfer cell (BioRad) during 8 min at 100 V and 350 mA. After blocking in 5% skim milk, the blot was probed with rabbit anti-DsrC from *D. vulgaris* followed by incubation with AP-conjugated anti-rabbit IgG (1:15,000; Sigma), as described in (Venceslau *et al.*, 2013).
3.3. Results and discussion

We report both in vitro and in vivo studies showing that DsrC is a substrate for DsrAB and that the product of dissimilatory sulfite reduction by DsrAB is a DsrC trisulfide formed between the two conserved Cys of its C-terminal arm. First we tested the effect of DsrC on the activity of sulfite reduction by DsrAB in vitro, looking at the oxidation of reduced methyl viologen (Mv) as artificial electron donor (Figure 3.4A).

For these studies we used DsrAB and DsrC from the thermophilic archaeon *Archaeoglobus fulgidus*, as DsrAB can be isolated from this organism separate from DsrC (Schiffer et al., 2008) (in contrast to Desulfovibrio organisms where the two proteins form a tight complex). The presence of reduced DsrC induced an initial fast rate of reaction, which then slows to a rate similar to that in experiments where DsrC is

![Figure 3.4](image.png)

*Figure. 3.4. Effect of DsrC on sulfite reduction by DsrAB. (A) Sulfite reduction activity of DsrAB in the presence of different DsrC concentrations. Activity is monitored by oxidation of Mv followed at 732 nm. Representative traces are shown. (B) Specific activity of DsrAB in the presence/absence of DsrC (7.5 μM). Data points are mean ±SD, N=3.*
absent. The duration of the fast phase is directly proportional to the amount of DsrC added (Figure 3.5).

Figure 3.5. Variation of the length of the fast phase of sulfite reduction by DsrAB with DsrC concentration.

During the fast phase, the sulfite reduction rate is increased by 15-fold over the slow phase at 60 °C (the temperature routinely used for the assay), and this effect is even more evident at 80 °C, the optimum temperature for this enzyme (Figure 3.4B). Thus, reduced DsrC induces a strong increase in the specific activity of sulfite reduction by DsrAB, while the $K_m$ for sulfite is not affected (11±4 µM in the absence of DsrC and 12±3 µM in its presence).

When the DsrC cysteines are previously alkylated or when DsrC is added in the oxidized disulfide state (Venceslau et al., 2013), then no effect on the reaction rate of DsrAB is observed. This indicates that the rate increase is strictly dependent on the presence of reduced Cys$_A$ and Cys$_B$ on DsrC. The fact that duration of the fast phase is dependent on the concentration of DsrC (Figure 3.5) suggests that it is being consumed (i.e. oxidized) in the reaction. To further test this we performed a
A DsrC trisulfide links dissimilatory sulfate reduction to energy conservation

sequential addition experiment where more DsrC was added after reaching the slow phase (Figure 3.6). Upon each addition of DsrC, another fast reaction phase occurred, where the duration was again proportional to the amount of DsrC added. This further confirms that the transition to the slow phase is induced by the DsrC-concentration dependence of sulfite reduction kinetics. Tracking sulfite consumption during the fast phase (upon addition of different DsrC concentrations), a clear one to one relationship (Figure 3.7) reveals that DsrC is being used as a substrate by DsrAB. We further quantified how much Mv is oxidized in the fast and slow phases relative to the concentration of sulfite consumed in the same period. This relationship is $1.8\pm0.3 \, \mu\text{mol Mv/\mumol SO}_3^{2-}$ in the fast phase and $4.1\pm1.2 \, \mu\text{mol MV/\mumol SO}_3^{2-}$ in the slow phase. Since Mv is a one-electron donor, this indicates that two electrons are being used to reduce sulfite in the presence of DsrC. In the absence of DsrC the number of electrons is higher and more variable, which

Figure. 3.6. Effect of DsrC on sulfite reduction by DsrAB. Sulfite reduction activity by DsrAB upon successive additions of DsrC. The reaction mixture contained reduced Mv and sulfite and at 20 s DsrAB (430 nM) and DsrC (7.5 µM) were added. After reaching the slow phase, two additions of DsrC were performed (at 43 s and 65 s).
agrees with the reported mixture of products being formed (trithionate, thiosulfate and sulfide), corresponding to different degrees of reduction. DsrAB can also reduce thiosulfate or trithionate (Parey et al., 2010), so we tested if DsrC has an effect on the rate of reduction of these two compounds. No effect was observed (Figure 3.8), suggesting these are not physiological substrates of DsrAB. Taken together, these results

![Graph](Image)

*Figure. 3.7. Effect of DsrC on sulfite reduction by DsrAB. Sulfite consumed in the fast phase versus the concentration of DsrC present in the assay. A sample of the reaction mixture was taken at the beginning and end of the fast phase for sulfite quantification by HPLC (all data points are mean ± SD, N=3).*

![Graph](Image)

*Figure. 3.8. Effect of DsrC on the reduction rate of different substrates with DsrAB. (A) reduction of sulfite; (B) reduction of thiosulfate; (C) reduction of trithionate. Oxidation of MV was monitored at 732 nm using 7.5 µM wild-type DsrC and 250 µM of substrate.*
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indicate that DsrC is a requisite substrate in physiological sulfite reduction and key to the dissimilatory sulfate/sulfite reduction pathway.

In order to identify the sulfur products formed during sulfite reduction by DsrAB in the absence / presence of DsrC, we tracked sulfide and thiosulfate (as fluorescent bromobimane derivatives) from the reaction mixture across both fast and slow phases. In the absence of DsrC, sulfide and thiosulfate are produced right from the start of the reaction (Figure 3.9A), whereas in the presence of DsrC (50 µM) no sulfur products are detected until the reaction enters the slow phase (Figure 3.9B). Similar results were observed for trithionate in experiments using

![Graph showing sulfite consumption and thiosulfate and sulfide production by DsrAB.](image)

Figure. 3.9 Sulfite consumption and thiosulfate and sulfide production by DsrAB. Sulfite, thiosulfate and sulfide were measured by HPLC after mbbr labelling from samples taken at different time points from the enzymatic assays. (A) Assay in the absence of DsrC; (B) Assay in the presence of 50 µM wild-type DsrC; (C) Assay in the presence of 50 µM DsrC CysBAla variant; (D) Assay in the presence of 50 µM DsrC CysBAla variant. (All data points are mean ±SD, N=3). The grey areas highlight the fast phase in the presence of WT and CysBAla DsrC.
a higher concentration of DsrC (100 µM). In parallel, the redox state of DsrC was analyzed during the fast and slow phases with a gel-shift assay using the cysteine-labelling reagent MalPEG, which increases the mass of the protein by about 10kDa per reduced Cys (Venceslau et al., 2013). Two parallel experiments were carried out with different concentrations of DsrC (50 and 100 µM) (Figure 3.10A). At time zero, DsrC is in a reduced state as revealed by the shift for one (CysA) and

Figure. 3.10. Redox state of DsrC upon reaction with DsrAB and sulfite. (A) Kinetic trace of Mv oxidation in two enzymatic assays with different DsrC concentrations: 50 µM (blue) and 100 µM (red). Samples were taken at the time points indicated for analysis of DsrC redox state. (B) Gel shift analysis of MalPEG-labelled DsrC taken from the 50 µM assay at different time points. Shifts for one and two Cys labelled are observed in the reduced state as CysA is more accessible for labelling than CysB. At the end of the fast phase all DsrC is converted to a form where no Cys is labelled (no shift). (C) Gel shift analysis of MalPEG-labelled DsrC taken from the 100 µM assay at different time points. Complete oxidation of DsrC is observed at 10 min versus 3 min in (B). (D) Gel shift analysis of MalPEG-labelled DsrC taken from a control assay where DsrAB is omitted. (E) Gel shift analysis of MalPEG-labelled DsrC taken from enzymatic assays with wild-type DsrC (WT), CysAAla and CysBAla variants. Samples were taken after reaching the slow phase.
two cysteines (Cys_A and Cys_B) labelled, but during the reaction an oxidized form is produced, as evidenced by no observed shift (Figure 3.10B and 3.10C). In both experiments, at the end of the fast phase all DsrC is converted to this oxidized form where the two Cys no longer react with MalPEG. In a control experiment where DsrAB is absent, DsrC remains reduced (Figure 3.10D).

Mass spectrometry analysis of intact DsrC recovered after the end of the fast phase (1530.7 Da), showed a distinct mass increase of 32 Da, relative to the value measured at the start of the experiment (1498.7 Da), indicating that one sulfur atom is bound to DsrC after reaction with DsrAB and sulfite. For increased mass accuracy, we performed peptide mass fingerprinting of DsrC recovered from the reaction, with and without alkylation, focusing on the C-terminal peptide (residues 101 to 115) (Table 3.1 and Figure 3.11). At time zero alkylation with iodoacetamide or DTNB confirmed the presence of two reduced Cys on DsrC (Figure 3.11A and 3.11C). After the fast phase, the mass of the DsrC C-terminal peptide shows a precise mass increase of 32 Da (Figure 3.11B), and no reactivity with the alkylating reagents (Figure 3.11D), indicating that Cys_A and Cys_B are no longer reduced. In control experiments where DsrAB is absent, the C-terminal peptide is not altered (Figure 3.12A and 3.12B), with two free Cys present before and after the incubation (Figure 3.12C and 3.12D). These results allow us to discount our prior model where we predicted a DsrC persulfide as the product of sulfite reduction by DsrAB (Oliveira et al., 2008, Venceslau et al., 2014); this is because a persulfide would react with both the alkylating and gel shift reagents, which is clearly not occurring. Moreover, chemical oxidation of a persulfide product is also highly unlikely under the strictly anaerobic conditions of the experiment. These results reveal the nature of the product of sulfite
reduction by DsrAB/DsrC as a DsrC-bound trisulfide species, where a single sulfur originating from sulfite is bound to the two DsrC cysteines (CysA and CysB).

Table 3.1. Mass spectrometry data of the DsrC C-terminal peptide. The mass (Da) of the $^{101}$DACRIAGLPKPTGCV$^{115}$ peptide was measured from digested DsrC (WT, CysAAla and CysBAla variants) recovered from the reaction mixture at time zero ($T_0$) and at the end of the assay ($T_F$). Before digestion the recovered DsrC was alkylated with iodoacetamide (+IA) or not (-IA). Nº Cys refers to the number of free Cys present in the sample as derived from the mass increase after reaction with iodoacetamide. The mass difference refers to the difference between the C-terminal peptide mass at the end of the assay and at time zero (without alkylation). In the control experiment DsrAB was omitted from the assay mixture.

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<th>$T_F$ - IA</th>
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<td>2</td>
<td></td>
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<td></td>
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</tr>
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<td>1</td>
<td></td>
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<tr>
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<td>1525,8</td>
<td>1468,8</td>
<td>1525,8</td>
<td>0</td>
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<td>1</td>
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Figure. 3.11. MALDI-TOF spectra of wild-type DsrC peptides before and after reaction with DsrAB and sulfite. The DsrC C-terminal peptide \( ^{10} \text{DACRIAGLPKPTGCV}^{15} \) with 1498.7 Da) before the reaction (A) shows a mass increase of 32 Da after the enzymatic reaction (B). A small amount of unreacted DsrC is detected. The number of free cysteines in DsrC before (C) and after (D) enzymatic sulfite reduction was assessed by alkylation with iodoacetamide, which adds 57 Da per each reduced cysteine. This shows that the 1530.7 peptide is not alkylated (D). Other peptides of no interest are marked with X.
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To further investigate the role of each C-terminal Cys in the reaction mechanism, we produced two DsrC variants lacking each of these residues. The protein lacking CysA (CysA-Ala variant) did not increase the rate of DsrAB-catalyzed sulfite reduction, but had a significant effect on the product composition; here sulfide is the major product with negligible production of thiosulfate or trithionate (Figure 3.9C). In contrast, the protein lacking CysB (CysB-Ala variant) had a similar effect on DsrAB activity as the wild-type DsrC, both in terms of rate and absence of free sulfur products in the fast phase (Figure 3.9D). In the gel shift assay the
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CysAAla variant showed a shift corresponding to one free Cys after the reaction (Figure 3.10E). Alkylation also confirmed one free Cys at the start and end of the experiment, and no mass increase was observed either by intact mass or peptide mass fingerprinting (Table 3.1 and Figure 3.13). In the gel shift assay the CysAAla variant showed a mixture of two products, one with no shift corresponding to an oxidized form and one with one free Cys (Figure 3.10E). Intact mass analysis of this variant showed a mass increase of 52Da, which is not alkylated, and agrees with a persulfide sulfenate (SOH) derivative of CysA. Peptide mass fingerprinting revealed the presence of two products, a major one with no mass increase, and a minor one with additional 32Da (Table 3.1 and Figure 2.14B). Alkylation revealed the presence of one free Cys at the start and end of the experiment (Table 3.1 and Figure 3.14D).
Figure 3.13. MALDI-TOF spectra of DsrC Cys₆Ala variant peptides before and after sulfite reduction by DsrAB. The Cys₆Ala DsrC C-terminal peptide (¹⁰⁷DACRIAGLPKPTGAV₁¹⁵ with 1468.8Da) (A), is not altered in the enzymatic reaction (B). The number of free cysteines in Cys₆Ala DsrC before (C) and after (D) enzymatic sulfite reduction was assessed by alkylation with iodoacetamide. Other peptides of no interest are marked with X.
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Figure. 3.14. MALDI-TOF spectra of DsrC Cys$_B$Ala variant peptides before and after reaction with DsrAB and sulfite. The Cys$_B$Ala DsrC C-terminal peptide (DAARIAGLPKPTGCV with 1468.8Da) before the reaction (A) presents two forms after the enzymatic reaction, an unaltered form and a persulfide form (1500.8Da corresponding to a +32Da increase). The number of free cysteines in Cys$_B$Ala DsrC before (C) and after (D) enzymatic sulfite reduction was assessed by alkylation with iodoacetamide. Other peptides of no interest are marked with X.

Taken together these results suggest a mechanism for the DsrAB/DsrC reduction of sulfite (Figure 3.15). Oxidized DsrAB takes up two electrons from a physiological electron donor reducing the siroheme Fe and its coupled [4Fe-4S] cluster (step 1). Following binding of bisulfite (the most likely substrate at physiological pH), a subsequent two-electron reduction and dehydration takes place to produce an $S^\text{II}$ intermediate (steps 2 and 3) (Crane et al., 1997, Parey et al., 2010). This
is then reduced by DsrC Cys<sub>A</sub> concomitant with release of H<sub>2</sub>O to form an S<sup>I</sup> intermediate bound to DsrC (step 4). Structural rearrangement of the DsrC C-terminal arm, such that the two Cys come together, then

![Diagram of the reduction process](image-url)

Figure. 3.15. **Physiological role of DsrC in sulfite reduction by DsrAB.** (A) Proposed mechanism for reduction of sulfite by DsrAB with involvement of DsrC. The reaction is assumed to start with the two-electron reduction of the heme and [4Fe-4S] cluster of oxidized DsrAB (in blue) by a redox partner (step 1), followed by binding of bisulfite (step 2). These two steps may occur in the reverse order. A two-electron reduction of the substrate followed by dehydration leads to a S<sup>II</sup> intermediate (step 3). Reaction of this intermediate with DsrC Cys<sub>A</sub> and dehydration leads to the S<sup>I</sup> intermediate (step 4). We postulate that this intermediate is released from the heme Fe coordination, and that the DsrC C-terminal arm adopts the retracted conformation where the two Cys are in short distance from each other (step 5). This allows the reaction of Cys<sub>A</sub> with the S<sup>I</sup> intermediate, leading to production of the trisulfide product (in green, step 6).
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allows internal reduction by DsrC CysB, with H2O release, yielding the S0 trisulfide product (steps 5 and 6). Our proposed mechanism is supported by the observation that the DsrC C-terminal arm can adopt a retracted conformation while still bound to DsrAB (Hsieh et al., 2010), where the two Cys are in closer proximity to one another, allowing for the attack of CysB on the S1 intermediate. Notably, a direct disulfide bond between the two DsrC Cys has never been observed in the crystal structures reporting this retracted conformation of the DsrC C-terminal arm (Mander et al., 2005, Hsieh et al., 2010). Such a disulfide bond is likely sterically hindered, as supported by the difficulty in generating the internal CysA-CysB disulfide in vitro. In oxidizing conditions, a DsrC dimer connected by a CysA-CysA bond is produced instead (Venceslau et al., 2013).

This novel mechanism clearly explains the results obtained with the DsrC variants. In the absence of DsrC CysA, which is the first key Cys to

<table>
<thead>
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<th>CysAAla DsrC</th>
<th>CysBAla DsrC</th>
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<td>- No rate increase in sulfite reduction</td>
<td>- Rate increase similar to WT-DsrC</td>
</tr>
<tr>
<td>- No mass increase in sulfite reduction</td>
<td>- Mass increase of 52 kDa (intact) or 32 kDa (peptide)</td>
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<td>- DsrC product:</td>
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<td><img src="image2.png" alt="Image of CysBAla DsrC product" /></td>
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Figure. 3.16. Products of sulfite reduction by DsrAB and DsrC variants. The CysAAla variant has no effect on sulfite reduction and is not modified. The CysBAla variant has a similar effect as WT DsrC, but the products formed are unstable.
react, there is no effect on the reaction rate and DsrC is not modified (Figure 3.16).

In the absence of Cys8, the S\(^{I}\) intermediate with a persulfide sulfenate group is produced, which cannot react further and can be released as a product (corresponding to the detected mass increase of 52 Da). This is likely an unstable product and decomposes to a mixture of the persulfurated (+32 Da) and the unmodified protein, as detected by peptide mass fingerprinting and alkylation (Figure 3.14).

The \textit{in vitro} production of thionates (S\(_3\)O\(_6\)\(^{2-}\) and S\(_2\)O\(_3\)\(^{2-}\)) by DsrAB in the absence of reduced DsrC, can be explained by further reaction of the partially reduced S\(^{II}\) and S\(^{0}\) intermediates with more sulfite, yielding trithionate and thiosulfate, respectively (Figure 3.17).

**A**

Reduced DsrC is available:

![Diagram](image)

**B**

DsrC is not functional:

![Diagram](image)

\textit{Figure}. 3.17. \textit{Products of DsrAB sulfite reduction in the presence or absence of functional DsrC.} (A) If reduced DsrC is available DsrAB receives two electrons from a physiological partner and two other electrons are supplied by DsrC to form the trisulfide product. (B) If DsrC is not functional the S\(^{II}\) intermediate can react with additional sulfite to form trithionate, or it can be further reduced to give a S\(^{0}\) intermediate, which can also react with further sulfite to produce thiosulfate or be reduced further to give sulfide.
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It should be noted that in the absence of DsrC the siroheme active site of DsrAB is directly accessible through a wide channel where the DsrC C-terminal arm usually binds. This channel is distinct from the much narrower substrate access channel, where sulfite must diffuse in when DsrC is docked to DsrAB (Oliveira et al., 2008). This different substrate accessibility in the presence and absence of DsrC explains the product composition observed for the Cys\textsubscript{A}Ala DsrC variant (Figure 3.9C). With this variant the C-terminal arm inserts into the DsrAB structure blocking the DsrC channel (though not participating in the reaction due to the Cys\textsubscript{A}Ala mutation), with access of sulfite now occurring exclusively through the narrower substrate channel. This likely restricts or slows down the reaction of additional sulfite with the semi-reduced intermediates, allowing for its complete reduction and the observed production of sulfide as the main product (Figure 3.9C). Therefore, previous reports of thiosulfate and trithionate production as \textit{in vitro} products of DsrAB (Lee & Peck, 1971, Peck et al., 1982) are the result of \textit{in vitro} experimental parameters that do not represent physiological (\textit{in vivo}) conditions – e.g., the absence of reduced DsrC and high concentrations of (bi)sulfite.

To complement these results we did \textit{in vivo} studies on the physiological role of DsrC, using \textit{D. vulgaris}, a model sulfate reducing deltaproteobacterium for which genetic tools are available. Multiple attempts to produce a \textit{dsrC} deletion mutant proved unsuccessful, as also observed by others (J. D. Wall, personal communication), revealing that DsrC is an essential protein in this organism. We took a different approach for genetic studies of \textit{dsrC} in \textit{D. vulgaris} and provided a second copy of the \textit{dsrC} gene \textit{in trans} on a plasmid, resulting in strain IPFG06 (Figure 3.18).
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Figure 3.18. Schematic representation of the different *D. vulgaris* strains produced. The wild-type *D. vulgaris* (DvH) contains only the chromosomal copy of the dsrC gene (cDsrC). IPFG06 contains both the chromosomal and a plasmid copy of dsrC (pDsrC). IPFG07 contains only the plasmid copy of dsrC. IPFG09 contains only the plasmid copy of dsrC, containing the CysβAla mutation and a C26A mutation in one structural cysteine.

Figure 3.19. Western blot of DsrC in the different *D. vulgaris* strains. The plasmid-encoded DsrC (pDsrC) has a higher mass than the chromosome-encoded DsrC (cDsrC) due to the presence of a His-tag. The Western blot for DsrB in the same samples is shown as internal control.

This strain produces native chromosomally encoded DsrC (c-DsrC) plus a minor amount of plasmid encoded DsrC (p-DsrC) (Figure 3.19). The chromosomal dsrC gene was deleted in strain IPFG06 to generate strain IPFG07 containing only the plasmid dsrC copy transcribed from a weaker promoter (p-DsrC). The same strategy was applied to generate strain IPFG09 where only a plasmid dsrC copy encoding a Cysβ to alanine exchange is present (p-CysβAla-DsrC). Western blot revealed
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reduced levels of DsrC in strains IPFG07 and IPFG09, as expected (Figure 3.19). In the case of DsrC lacking CysA we generated the *D. vulgaris* strain with the intact chromosomal copy of dsrC and CysAAla-

![Figure 3.20](image-url)

Figure 3.20. (A) Growth profile of *D. vulgaris* wild-type (WT) and IPFG06, IPFG07 and IPFG09 strains on lactate/sulfate medium. The corresponding doubling times and maximal optical density are shown below. Data are mean ±SD, N=3. All differences are statistically significant (p-values≤0.001). (B) Sulfate consumption of the different *D. vulgaris* strains. Sulfate measurements were performed from growth experiments in (A) (30mM lactate/30mM sulfate). Data are mean ±SD, N=3.

dsrC in trans but, despite numerous attempts, it was not possible to delete the chromosomal copy and generate the strain with only CysAAla-dsrC, even with selection under pyruvate fermentation conditions. This strongly indicates that CysA is necessary for functional DsrC, and further, that CysA-containing DsrC is necessary for the growth of viable cells. We then compared the growth profiles of strains IPFG06, IPFG07 and IPFG09 during lactate/sulfate respiration to those of the wild-type *D. vulgaris* (Figure 3.20A). Strain IPFG06 showed a small increase in doubling time relative to the wild-type and reached similar cell densities (Figure 3.20A). In contrast, the strain containing only p-DsrC (IPFG07) had a significant increase in doubling time and reached a lower cell density. This effect was even more pronounced for the strain containing p-CysBAla-DsrC (IPFG09) (Figure 3.20A). The differences in growth rate
were also reflected in sulfate consumption rates (Figure 3.20B). Overall, these \textit{in vivo} studies showed that DsrC and specifically its Cys\textsubscript{A} are essential for sulfate reduction, as well as cell viability. Furthermore, it is clear that Cys\textsubscript{B}, though not strictly essential, plays an important role in sulfite reduction. Moreover, the overall expression level of DsrC is a limiting factor in sulfate respiration, as revealed by the reduced growth rate of strain IPFG07.

\subsection*{3.4. Conclusion}

Overall, our \textit{in vitro} and \textit{in vivo} results provide evidence that DsrC is an essential protein for sulfite reduction by DsrAB and thus for sulfate/sulfite respiration. DsrC is in fact a substrate for DsrAB and, along with sulfite, is converted to the trisulfide form over the course of the reaction. Protein polysulfides and persulfides have recently emerged as the cellular currency for an activated form of sulfur that is involved in key cellular functions, such as H\textsubscript{2}S signaling, biosynthesis of sulfur-containing cofactors, and modulation of enzyme activities and redox homeostasis (Paul & Snyder, 2012, Ida \textit{et al}., 2014, Toohey & Cooper, 2014). Our data expand the physiological role of these sulfur species by showing that a protein trisulfide is a key metabolite in the dissimilatory sulfate reduction pathway. Protein trisulfides have also been reported in proteins involved in tRNA sulfuration (Liu \textit{et al}., 2012, Liu \textit{et al}., 2012), in sulfide:quinone oxidoreductase (Cherney \textit{et al}., 2012) and in several antibodies (Nielsen \textit{et al}., 2011). The finding of a trisulfide contrasts with our previous proposal that DsrC persulfide and disulfide forms would be involved in sulfite reduction (Oliveira \textit{et al}., 2008, Venceslau \textit{et al}., 2014), but is actually a superior solution, both in chemical and biological terms. Our new mechanism indicates that upon (bi)sulfite reduction by DsrAB only two electrons are taken up by DsrAB from an, as yet unidentified,
A DsrC trisulfide links dissimilatory sulfate reduction to energy conservation. The other two electrons necessary to produce the trisulfide originate from Cys\textsubscript{A} and Cys\textsubscript{B} on reduced DsrC. Critically, the DsrC trisulfide will be reduced in a fourth step to produce the final product sulfide and recycle DsrC. This new step in the sulfate reduction pathway is most likely carried out by the membrane DsrMKJOP complex (Figure 3.21), which includes the DsrK subunit whose characteristic EPR signature indicates a \([4\text{Fe}-4\text{S}]\) cluster known to perform disulfide reductions (Pires \textit{et al.}, 2006, Grein \textit{et al.}, 2013, Venceslau \textit{et al.}, 2014). DsrK is closely related to the HdrD heterodisulfide reductase of methanogens (Hedderich \textit{et al.}, 2005), and has been shown to interact with DsrC (Grein \textit{et al.}, 2010, Venceslau \textit{et al.}, 2014).

\textbf{Figure. 3.21. \textit{Schematic representation of the sulfate respiratory pathway}, involving import of sulfate into the cell, its activation by sulfate adenylyl transferase (Sat) to adenosine 5'-phosphosulfate (APS) (I), reduction of APS to sulfite by the APS reductase (II), reduction of sulfite to the DsrC trisulfide by DsrAB/DsrC (III), and finally reduction of the DsrC trisulfide to sulfide and reduced DsrC by the DsrMKJOP membrane complex (IV).}
The existence of a fourth step in the dissimilatory sulfate reduction pathway has far reaching implications for the bioenergetics of this anaerobic respiratory process. It means that four of the six electrons for (bi)sulfite reduction originate from the reduced menaquinone pool, coupling the generation of a ΔpH across the membrane directly to sulfite reduction and energy conservation. Therefore, the production of a DsrC trisulfide, and subsequent reduction by DsrK, allows for the soluble process at DsrAB to be coupled to energy conservation at the membrane level (DsrMKJOP). These insights into the mechanism of biological sulfate reduction carry significant and unexplored consequences for interpreting reaction rates and isotope effects in sulfur metabolism and improve our understanding of the sulfur cycle, both in modern and in ancient times.

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Chapter 4

Searching for the DsrAB physiological electron donor
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4.1. Introduction

We showed in the previous chapter that four of the six electrons needed for sulfite reduction by DsrAB are donated by DsrC, but the physiological partner that donates the first two electrons has still not been identified.

The DsrAB protein contains two ferredoxin like domains per each αβ structure (in DsrB), each one containing an exposed [4Fe-4S] cluster that is suggested to receive electrons from an external donor and transfer them to the catalytic site (Figure 4.1) (Dahl et al., 1993, Karkhoff-schweizer et al., 1995, Oliveira et al., 2008). Based on this information we reasoned that the physiological electron donor to DsrAB should be also a ferredoxin or a ferredoxin-reducing protein.

In 1999 Larsen et al identified a ferredoxin-like gene immediately upstream of dsrA gene in Desulfotomaculum thermocisternum. With a multiple alignment of 23 [4Fe-4S]–ferredoxins, they grouped this
ferredoxin from *D. thermocisternum* along with ferredoxin IV of *A. fulgidus* and ferredoxin I from *D. vulgaris* (Larsen et al., 1999). After, in 2011 Pereira and coworkers confirmed that all SRP have one or several copies of ferredoxin I, and that in three Gram-positive organisms and *Ammonifex degensii*, the *dsrMK-dsrC* gene cluster is preceded by a ferredoxin gene. Moreover, a ferredoxin gene was also found after the *dsrMKJOP* genes and in close proximity to *dsrAB* in three *Deltaproteobacteria* (Pereira et al., 2011). These observations support the proposal that a ferredoxin is involved in the same metabolic pathway as DsrAB, and can possibly act as its physiological electron donor.

We analyzed the genome of several SPR looking for ferredoxin-reducing enzymes that would be present in all SRP. Three possible candidates were selected: the aldehyde oxidoreductase (AOR), the pyruvate ferredoxin-oxidoreductase (PFOR) and the carbon monoxide dehydrogenase (CODH).

**Aldehyde oxidoreductase (AOR).** Prokaryotic AORs are either mononuclear molybdenum-containing enzymes binding one pyrrolopterin from the xanthine oxidase family, or tungsten-containing enzymes that have a bis-pyrrolopterin cofactor and use ferredoxin as

![Figure. 4.2. Active-site structures of molybdenum- and tungsten-containing AORs and the structure of the pyrrolopterin cofactor common to molybdenum- and tungsten-containing AORs (Hille et al., 2014).](image-url)

\[ RCHO + H_2O + 2Fd_{ox} \rightarrow RCOOH + 2H^+ + 2Fd_{red} \ (4.1) \]

All purified tungsten-containing AORs are highly sensitive to oxygen, losing activity immediately after contact with oxygen (Mukund & Adams, 1991, Hensgens et al., 1995, Roy et al., 2001, Rauh et al., 2004, Bevers et al., 2005), while the molybdenum-containing proteins are tolerant to oxygen (Barata et al., 1993, Thapper et al., 2006). Also, the tungsten-containing AORs have an aldehyde activity around one order of magnitude higher than the molybdenum-containing AORs. It was described by Hensgens and coworkers that D. gigas probably has both enzymes depending on the metal (Mo or W) used during growth (Hensgens et al., 1994).

**Pyruvate ferredoxin-oxidoreductase (PFOR).** PFORs have a variable quaternary structure as they are mostly homodimeric but with examples of the heterodimeric form (such as in Halobacterium PFOR enzymes (Ragsdale, 2003) or octomeric form (D. vulgaris Hildenborough (Garczarek et al., 2007)). PFORs are thiamine pyrophosphate (TPP)-containing iron-sulfur proteins that catalyze the oxidative decarboxylation of pyruvate with coenzyme-A and with ferredoxin (or flavodoxin) as the electron acceptor (equation 4.2) (Uyeda & Rabinowitz, 1971):

\[ C_3H_4O_3 + CoA + Fd_{ox} \rightarrow acetyl-CoA + Fd_{red} \ (4.2) \]
Due to its very low redox potential (-540 mV), this reaction plays a crucial role in anaerobic microorganisms, as it can achieve the reduction of ferredoxin or flavodoxin. Even further, in strict anaerobes, PFOR allows for energy conservation by substrate-level phosphorylation through the phosphoroclastic reaction (equation 4.2-4.4) (Ragsdale, 2003).

\[
acetyl-CoA + Pi \rightarrow CoA + acetyl-phosphate \tag{4.3}
\]

\[
acetyl-phosphate + ADP \rightarrow ATP + acetate \tag{4.4}
\]

**Carbon monoxide dehydrogenase (CODH).** CODHs are proteins able to catalyze the (reversible) oxidation of CO with water to CO\(_2\) (equation 4.5) (Jeoung et al., 2014). CODHs proteins can fall into two classes: while in aerobic bacteria CODHs contain a copper and molybdenum active site, anaerobic bacteria and archaea use a nickel and iron-containing CODH (Jeoung et al., 2014).

\[
CO + H_2O \rightleftharpoons CO_2 + 2H^+ + 2e^- \tag{4.5}
\]

CODHs have multiple biological roles where they are associated with different physiological partners. CODHs can be associated with energy conservation as they can be coupled with membrane bound hydrogenases to generate a proton motive force by combining the oxidation of CO with the production of hydrogen (Soboh et al., 2002, Wu et al., 2005). Also, CODHs can be linked to the Wood-Ljungdahl pathway where a Ni,Fe-CODH forms a complex with acetyl-CoA synthase (ACS),
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in which CO₂ is reduced to CO at the catalytic center of CODH and used by ACS coupling it with a methyl group to form acetyl-CoA (Ragsdale, 2004). Moreover, CODHs can be associated with oxidative stress response and with the generation of NADPH (Wu et al., 2005).

**NADH oxidoreductase.** NADH oxidoreductases are able to (reversibly) oxidize NADH with an electron acceptor to NAD⁺ and protons (equation 4.7).

\[
NADH + e^- + acceptor \rightleftharpoons NAD^+ + H^+ \quad (4.7)
\]

Membrane-bound NADH:quinone oxidoreductases can be divided in three different families: Complex I, Type II NAD(P)H dehydrogenase (NDH-2) and Na⁺-translocating NADH:quinone oxidoreductase complex (Na⁺-NQR) (Feng et al., 2012). The respiratory Complex I, present in many aerobic organisms, couples the oxidation of NADH to the reduction of quinone or ubiquinone and exports protons to the periplasm creating a proton motive force crucial for the generation of ATP by ATP synthases (Sazanov, 2015). The NDH-2 can be found in prokaryotes, fungi and plants, and can catalyze the two-electron transfer from NAD(P)H to quinones, without the translocation of protons across the membrane (Melo et al., 2004). The third family is the rarest, the Na⁺-NQR, present in some bacteria, are able to couple the transport of Na⁺ with the transfer of electrons from NADH to quinone/ubiquinone, generating a redox-driven transmembrane electrochemical Na⁺ potential (Barquera, 2014, Reyes-Prieto et al., 2014). Na⁺-NQR is evolutionary related to Na⁺-dependent NADH:ferredoxin oxidoreductase, Rnf complex, present in several anaerobes (Biegel & Muller, 2010), which links the oxidation of
NADH to the reduction of ferredoxin, importing Na\(^+\)/H\(^+\) to the cytoplasm, or the reduction of NAD\(^+\) to the oxidation of ferredoxin to translocate Na\(^+\)/H\(^+\) across the cytoplasmic membrane generating a Na\(^+\)/H\(^+\) gradient that can be used to produce ATP (Schmehl et al., 1993, Biegel et al., 2011). Other NADH oxidoreductases are also soluble proteins with the ability to interact with different partners. Some of these proteins can be involved in electron bifurcation/confurcation (Figure 4.3) such as: the NADH-dependent reduced ferredoxin:NADP\(^+\) oxidoreductase (NfnAB), that uses NADH and reduced ferredoxin as electrons to reduce NADP\(^+\) (Wang et al., 2010, Huang et al., 2012); the electron-transferring flavoprotein (EtfAB), which in anaerobic bacteria can, for example, be coupled with lactate dehydrogenase linking the reduction of NAD\(^+\) with the oxidation of ferredoxin to reduce lactate to pyruvate (Weghoff et al., 2015); and the Flavin oxidoreductase (FlxABCD) that was shown to be associated with the heterodisulfide reductase complex (HdrABC) (Pereira et al., 2011) in which the reduction of a disulfide bridge and the reduction of a ferredoxin is linked to the oxidation of NADH (or the
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reverse) (Ramos et al., 2015). The NADH oxidoreductases can also be linked to oxygen scavenging mechanisms by rubredoxin:oxygen oxidoreductase (Roo). In this mechanism, the NADH-rubredoxin oxidoreductase oxidizes NADH and reduces rubredoxin, which will reduce Roo (a protein responsible for the reduction of oxygen to water without the formation of hydrogen peroxide (Chen et al., 1993, Santos et al., 1993)).

It is also known that NADH oxidases play an important role in sulfur oxidation and sulfite assimilation. According to Lübbe et al. when purifying the “reverse” DsrAB from A. vinosum another protein is always co-purified, a NAD\(^+\) reductase – DsrL (Lubbe et al., 2006). Furthermore, a mutant of A. vinosum containing a deletion in the drsL gene was completely unable to oxidize sulfur globules. This suggested a crucial role of DsrL in the oxidation of sulfur globules. Also, in the case of the aSiR, the electrons for sulfite reduction come from the flavoprotein subunit that has two flavin cofactors and accepts electrons from NADPH (Crane & Getzoff, 1996). These results led us to consider also an NADH oxidase as a possible electron donor for DsrAB for all SRP.

For these studies DsrAB from D. vulgaris and A. fulgidus were used. It must be noted that the pure fraction of DsrAB from D. vulgaris is always a mixture between \(\alpha_2\beta_2\), \(\alpha_2\beta_2\gamma\) and \(\alpha_2\beta_2\gamma_2\). On the other hand, the pure DsrAB fraction from A. fulgidus is only \(\alpha_2\beta_2\), having no DsrC protein covalently bound to DsrAB.
4.2. Materials and methods

Pyruvate ferredoxin-oxidoreductase (PFOR) from \textit{A. fulgidus}

The cultivation of \textit{A. fulgidus} VC16 (DSM 4304) was carried out as previously described (Stetter \textit{et al.}, 1987) with some changes: the yeast extract was 0.05% (w/v), growth at 83 °C, the flasks were flushed only with argon, the medium was buffered with 20 mM PIPES sodium salt, and 0.5 mM of sodium sulfide was used as initial reductant. The frozen cells were resuspended anaerobically in 20 mM potassium phosphate (Kpi) buffer pH 7 (buffer A) with 10% glycerol and 2 mM dithiothreitol, homogenized and disrupted in a French Press. The lysate was centrifuged at 58545 \times g for 20 minutes and the supernatant was centrifuged again at 100000 \times g for 2 hours. The purifications were performed in an anaerobic chamber. The soluble fraction was applied to a Q-Sepharose Fast Flow (2.6 cm × 10.0 cm; Amersham Pharmacia Biotech) equilibrated with buffer A. PFOR was eluted using a stepwise gradient (0-1 M KCl) at 0.25 M KCl. This fraction was desalted by ultrafiltration (cutoff = 10 kDa; Amicon) with subsequent dilution with buffer A. The desalted protein was loaded on a second Q-Sepharose Fast Flow (2.6 cm × 10.0 cm; Amersham Pharmacia Biotech) equilibrated with buffer A and eluted by a stepwise gradient (0-1 M KCl) at 0.25 M KCl. This fraction was desalted by ultrafiltration (cutoff = 10 kDa; Amicon) with subsequent dilution with buffer A. Finally this fraction was loaded on a phenyl-sepharose column (1.6 cm × 10.0 cm; Amersham Pharmacia Biotech) equilibrated with buffer A with 1.1 M of ammonium sulfate (buffer C). The PFOR fraction was eluted at 35% buffer A. The protein was concentrated to 0.7 mg/ml and maintained anaerobic and on
ice during use. The PFOR activity was measured in the anaerobic chamber using a spectrophotometer at 578 nm in a quartz cuvette with stirring. For this, 50 mM of Kpi pH 7 with 0.1 mM of Coenzyme-A, 5 mM of sodium pyruvate, 1 mM Dithiothreitol and 100 µL of sample where used.

**Aldehyde ferredoxin-oxidoreductase (AOR) from *D. gigas***

The cultivation of *D. gigas* was carried out as previously described (Barata et al., 1993) with pyruvate and 1 µM molybdenum and absence of tungsten. The frozen cells were re-suspended aerobically in 20 mM TrisHCl buffer pH 7.5 (buffer A), homogenized and disrupted in a French Press. The lysate was centrifuged at 58545 × g for 20 minutes and the supernatant was centrifuged again at 100000 × g for 2 hours. The soluble fraction was applied to a Q- Sepharose Fast Flow (1.6 cm × 10.0 cm; Amersham Pharmacia Biotech) equilibrated with buffer A. Aor was eluted in a stepwise gradient (0–1.0 M NaCl) at 0.25 M NaCl. This fraction was desalted by ultrafiltration (cutoff = 10 kDa; Amicon) with subsequent dilution with buffer A. The desalted protein was loaded on a Resource-Q 6 mL (Amersham Pharmacia Biotech) equilibrated with buffer A and eluted by a stepwise gradient (0 – 1 M NaCl) at 0.2 M KCl. This fraction was desalted by ultrafiltration (cutoff = 10 kDa; Amicon) with subsequent dilution with buffer A. Protein was concentrated to 13 mg/ml and maintained anaerobic and on ice during use. The purification of Aor was followed by aldehyde oxidase activity. This activity was performed aerobically in a spectrophotometer at 600 nm in a quartz cuvette with stirring. For this, 50 mM of TrisHCl pH 7.5 with 35 µM of DCPIP, 500 µM of acetaldehyde and 2 µL of sample where used.
Carbon monoxide dehydrogenase (CODH) from *D. vulgaris*

This enzyme was kindly given to us by Doctor Sébastien Dementin Principal Investigator at CNRS - Centre National de la Recherche Scientifique. The enzyme was produced and purified by Jessica Hadj-said a PhD student at Doctor Sébastien Dementin laboratory. For the CODH activities measure we used the following protocol: the enzyme was incubated at room temperature for 20 min in 1.5 mL tubes containing 50 µL of 0.44 µM enzyme solution in 0.1 M TrisHCl pH 8 in the presence of 2.4 mM of Ni + 1.2 mM of sodium dithionite. The CO oxidation activity assays were performed in a glove box (under N₂ atmosphere) using a spectrophotometer. CO oxidation was assayed at 37 °C by monitoring the reduction of methyl viologen (Mv) at 732 nm (ε = 3.15 mM⁻¹.cm⁻¹) in a quartz cuvette of 1 cm of optical length. The reaction was initiated by injecting 10 µL of pre-incubated enzyme in a stirred 1 mL solution containing 0.1 M TrisHCl pH 10, 2.4 mM of Mv and 25 µM of CO (25 µL of a CO-saturated solution injected just before enzyme addition) (Hadj-Said et al., 2015). This enzyme was always kept on ice inside an anaerobic flask next to a paper soaked in a sodium dithionite solution due to its immediate inactivation by oxygen.

Recombinant NADH oxidase (rNoxA-3) from *A. fulgidus*

The *A. fulgidus* VC16 noxA-3 gene (AF0400) was amplified by PCR using genomic DNA and the following oligonucleotides: 5’- ACC CCC ATA TGA ACG TTG TTG TAA TCG G -3’ and 5’- TTA CTC GAG TCA AAA CCA GAA GGC CAT TC -3’ with Ndel and Xhol restriction sites, respectively. The PCR product was cloned into pET22b(+) vector (Novagen), in which the native codon stop was maintained to remove the
strep tag. The recombinant plasmids were transformed in *E. coli* BL21 Gold (DE3) cells (Stratagene) containing the pRARE2 vector (for contains codons rarely used in *E. coli*) and the cells were grown at 37°C in LB medium (Roth Diagnostics) along with 6.3 μM Riboflavin, with ampicillin (100 μg/mL) and chloramphenicol (30 μg/mL) until an OD$_{600}$ of 0.4. At this stage 500 μM of isopropyl-b-D-thiogalactopyranoside (IPTG) was added and growth was continued for another 3h. Cells were then collect by centrifugation at 6000 × g for 10 minutes and stored at -20 °C for short periods or -80 °C for long time preservation. Cells were re-suspended in 20 mM Kpi pH 7 (buffer A) and disrupted in a French Press in the presence of a protease inhibitor cocktail (Complete by Roche Diagnostics). The extract was centrifuged for 20 min at 58545 × g, followed by a protein precipitation step at 70 °C and centrifugation for 5 min at 58545 × g. The soluble fraction was filtered and loaded on a Resource-Q 6 mL (Amersham Pharmacia Biotech) equilibrated with buffer A and eluted by a stepwise gradient (0-1 M KCl) at 0.350 M KCl. This fraction was desalted by ultrafiltration (cutoff = 10 kDa; Amicon) with subsequent dilution with buffer A. The protein was concentrated to 33 mg/ml and maintained anaerobic and on ice during use. The rNoxA-3 activity was followed by NADH oxidation. This activity was performed aerobically in a spectrophotometer at 340 nm in a quartz cuvette with stirring. For this, 50 mM of Kpi pH 7 with 100 μM of NADH, 5 μM of FAD and 2 μM of sample were used and the oxidation of NADH was monitored at 340 nm or at 600 nm, when DCPIP (80 μM) was used as the artificial electron acceptor.
Electron transfer assays and HPLC data analysis

The reactions of DsrAB with the possible electron donors were performed in an anaerobic chamber using 0.5 mL tubes at 37 °C for DsrAB from *D. vulgaris* and 60 °C for DsrAB from *A. fulgidus*. All activities were performed according to the electron donor reactions in the presence of DsrAB (0.430 μM) and sulfite (0.5 mM for DsrAB from *A. fulgidus* and 2.5 mM for DsrAB of *D. vulgaris*) as the electron acceptors. The decrease of sulfite was monitored by HPLC after derivatization with monobromobimane (mBbr). The mBbr stock was diluted in HPLC ultrapure acetonitrile to a final concentration of 50 mM. For the derivatization protocol, at each desired time 10 μL of sample was derivatized with a twofold excess of mBbr versus the initial concentration of sulfite, in HEPES buffer 20 mM pH 8, in a final volume of 86 μL (example: to 10 μL of sample with 500 μM of sulfite 1 mM of mBbr (2 μL) and 84 μL HEPES were added). The reactions were performed in the dark during 10 min after which 4 μL of methanosulfonic acid 5 M were added to stop the reaction. For HPLC separations the derivatized samples were diluted to the desired final concentration in 10 mM methanosulfonic acid. The analysis were executed using an HPLC (Waters Alliance 2695) equipped with a fluorescence detector (Waters 486) with excitation at 380 nm and emission at 480 nm, using a Beckman Coulter reversed-phase-column ultrasphere C18 Beckman Coulter (4.6-25; 5 μm), at 35 °C. The gradient was performed with 0.25% acetic acid pH 4 in MilliQ water (A) and 100% Methanol (B). Flow-rate was 1.20 mL/min. The elution protocol was as follows: 0-5 min 20% B, 5-13 min 20%-50% B, 13-16 min 50%-52% B, 16-20 min 52%-100% B, 20-24 min 100% B, 24-26 min 100%-20% B and 26-31 min 20% B. Calibration curves were performed with sodium sulfite.
Surface plasmon resonance (SPR) analysis

To analyze in vitro protein-protein interactions we used surface plasmon resonance in a BIAcore 2000 system. For the immobilization of the proteins the CM5 chip (carboxymethylated dextran covalently attached to a gold surface) was used, to which proteins bind via amine, thiol, aldehyde or carboxyl groups. All assays were performed at 25 °C with the running buffer (RB) (10 mM HEPES, 150 mM NaCl, 50 µM EDTA, 0.005% Tween 20 pH 7.4). DsrAB from *D. vulgaris* and *A. fulgidus* were covalently immobilized to the CM5 sensor chip by the amine coupling protocol described by the manufacturer. The proteins DsrAB from *D. vulgaris* and *A. fulgidus* were immobilized in the flow cells 2 and 3 respectively, in order to achieve 600 Resonance Units (RU) per which protein. The kinetic experiments were performed immediately after immobilization. Flow cell 1 was used as control. 500 mM NaCl was used to dissociate the analyte and regenerate the chip after each interaction assay. In all experiments the analyte was injected at a flow rate of 10 µM/min for 2 min over the flow cells 1, 2 and 3. After 2 min regeneration with NaCl, RB was applied to restore the base line.
4.3. Results and discussion

Electron donor analysis

Pyruvate ferredoxin-oxidoreductase (PFOR)

In this work we first tried to purify PFOR from *A. fulgidus* due to its presumed stability to high temperatures and to test for the interaction with DsrAB from the same organism. However, this enzyme proved to be extremely unstable, and after the purification we had very little protein and the activity rapidly decreased to 19% of the initial value. This led us to use instead the PFOR from *D. africanus*, a more stable protein, which was already available in our laboratory (purified as described by Pieulle and coworkers (Pieulle *et al.*, 1995)). In the structure of *D. africanus* PFOR, the TPP and a proximal [4Fe-4S] cluster are buried within the protein and the two additional [4Fe-4S] are placed near the surface which allow the interaction with a partner such as ferredoxin (Chabriere *et al.*, 1999). The PFOR from *D. africanus* is one of the few examples of a PFOR stable in the presence of oxygen. According to Chabrière and coworkers, this PFOR has an additional domain (domain IV), absent in many PFORs, which protects the enzyme against oxygen damage. This is a homodimeric protein with 256 kDa and three [4Fe-4S] clusters per subunit. Through sequence analysis of the α (H585DRAFT_03763) and β subunits (H585DRAFT_03764) we verified that: for the α subunit the identities with PFOR from *D. vulgaris* and *A. fulgidus* VC16 are low (E value of $2e^{-25}$ for *D. vulgaris* Hildenborough PorA α subunit, Dvu1569 and $3e^{-21}$ for *A. fulgidus* VC16 PFOR α subunit, AF0749); for the β subunit the E value is $4e^{-141}$ (PorB β subunit, Dvu1570) for *D. vulgaris* Hildenborough and $1e^{-22}$ (PFOR β subunit, AF0750). Although the PFOR from *D. africanus* only share high similarity with PorB.
of *D. vulgaris*, this is still a good candidate to test due to its ability to reduce ferredoxin like subunits.

We analyze the PFOR from *D. africanus* with circular dichroism for its stability at 60 °C, the temperature used for the *A. fulgidus* DsrAB activity. This allowed us to conclude that the structure of PFOR from *D. africanus* was still stable at this high temperature. The specific activity of our PFOR was of 17 U/mg in accordance with previous studies (Pieulle *et al.*, 1995)

**Aldehyde oxidoreductase (AOR)**

As referred before, to select the Mo-AOR, *D. gigas* must be grown on molybdenum and absent of W. We did not try to isolate the tungsten AOR due to its reported extreme oxygen sensitivity. Therefore, we grew *D. gigas* only in the presence of Mo and purified the corresponding AOR protein aerobically.

*Table 4.1. Sequence comparison between Aor genes of D. gigas and the homologues in D. vulgaris and A. fulgidus (analysis conducted in www.img.jgi.doe.gov)*

<table>
<thead>
<tr>
<th>D. gigas loci</th>
<th>D. vulgaris loci</th>
<th>A. fulgidus loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGI_0902</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DGI_1056</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td>DGI_1447</td>
<td>-</td>
<td>1e-08</td>
</tr>
<tr>
<td>DGI_3127</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E value</th>
<th>DVU1559</th>
<th>DVU1179</th>
<th>DVU0687</th>
<th>DVU0687</th>
<th>AF2281</th>
<th>AF0023</th>
<th>AF0077</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGI_0902</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DGI_1056</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>7e-78</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DGI_1447</td>
<td>-</td>
<td>-</td>
<td>1e-08</td>
<td>-</td>
<td>-</td>
<td>1e-17</td>
<td>-</td>
</tr>
<tr>
<td>DGI_3127</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>3e-137</td>
</tr>
</tbody>
</table>

Until the present day, only one AOR from *D. gigas* was found be resistant to oxygen and to contain Mo as the metal cofactor in *D. gigas* (DGI_0902). Moreover, from the four AOR present in *D. gigas*
(Table 4.1) three have an approximated weight of 60 kDa and only one (DGI_0902) of 100 kDa. Due to the fact that our AOR is resistant to oxygen (its activity did not decreased with time in the presence of oxygen) and that according to the 12% SDS gel the purified AOR has ~100 kDa, we know that our purified AOR is the DGI_0902. To measure the activity of AOR we used acetaldehyde and DCPIP as electron donor. After optimization of the protocol for the activity, this protein revealed a turnover of 2.71 s\(^{-1}\), which is about 2 times higher than the reported activity for the molybdenum AOR of *D. gigas* (Barata et al., 1993).

**Carbon monoxide dehydrogenase (CODH)**

The Ni,Fe-CODH used in this work is the CooS from *D. vulgaris* and it is predicted to have four [4Fe-4S] clusters and a mass of 67 kDa. The sequence comparison between the CODH of *D. vulgaris* and the CODH of *A. fulgidus* (AF_1849) shows a high similarity (E value 8e\(^{-125}\)). After incubation with nickel and dithionite, the CODH has an activity of 163 U/mg.

**Recombinant NoxA-3 from A. fulgidus VC16**

To search for conserved NADH oxidases in the SRP group we used all six NADH oxidases present in *D. vulgaris* Hildenborough (Dvu1613, DVU1165, DVU1974, DVU3212, DVU3292 and DVU2680) and blasted them against the list of SRP (using IMG). The locus DVU3212 was present in all 97 genomes analyzed with extremely high similarity, the only exception being *Thermodesulfovibrio yellowstonii* (no gene similarity). Homologues to this gene are also present in sulfite reducers, sulfur reducers and in organisms related to sulfur respiration such as *Pyrococcus furiosus* (PF1186). As so, we compared the gene locus DVU3212 against all five NoxA loci for *A. fulgidus* and identified the gene
locus AF0400 with the highest E value (Table 4.2). From this, we decided to use the noxA-3 gene to test as electron donor for DsrAB, as this is the most homologous protein to the widespread Nox in SRP. For this, the noxA-3 gene (AF0400) was cloned into a pet22b vector (without the tag to avoid disturbing the folding of the native NoxA-3).

Table 4.2. Analysis of the comparison of the DVU3212 gene locus from D. vulgaris with the five noxA gene loci present in A. fulgidus.

<table>
<thead>
<tr>
<th></th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D. vulgaris locus</strong></td>
<td><strong>A. fulgidus loci</strong></td>
</tr>
<tr>
<td>NoxA-1</td>
<td>AF0254</td>
</tr>
<tr>
<td>NoxA-2</td>
<td>AF0395</td>
</tr>
<tr>
<td>NoxA-3</td>
<td>AF0400</td>
</tr>
<tr>
<td>NoxA-4</td>
<td>AF0951</td>
</tr>
<tr>
<td>NoxA-5</td>
<td>AF1858</td>
</tr>
</tbody>
</table>

This vector was inserted in BL21 (DE3) Gold cells along with the pRARE2 vector for the production of rNoxA-3 protein. To purify this, a heat-shock treatment was first used to remove most of the E. coli native proteins that precipitate at high temperature (70 °C). After two ionic exchange columns, high purity rNoxA-3 was obtained. Since this was the first time that this protein has been isolated we characterized its activity.

The NoxA-3 protein from A. fulgidus VC16 is predicted to belong to Pyridine nucleotide-disulfide oxidoreductase, NAD-binding domain (Pfam PF00070), Rhodanese-like domain (SUPERFAMILY SSF52821) and to have a FAD/NAD(P)-binding domain. In order to verify which flavins are preferably bound by rNoxA-3, FAD, FMN or both, we checked the activity of rNoxA-3 alone or after addition of FAD, FMN or both at 37°C and 60 °C (Table 4.3).
Table 4.3. Activity (µmols of DCPIP reduced per minute per mg of enzyme) of rNoxA-3 with and without addition of flavins (5 µM) at 37 °C and 60 °C. Experiments performed with DCPIP as the artificial electron donor, 25 µM of NADH in Kpi 50 mM pH 7.

<table>
<thead>
<tr>
<th>Activity U/mg</th>
<th>37 °C</th>
<th>60 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNoxA-3</td>
<td>0.020</td>
<td>-</td>
</tr>
<tr>
<td>rNoxA-3 + FAD</td>
<td>0.101</td>
<td>1.540</td>
</tr>
<tr>
<td>rNoxA-3 + FMN</td>
<td>0.028</td>
<td>-</td>
</tr>
<tr>
<td>rNoxA-3 + FAD+FMN</td>
<td>0.096</td>
<td>1.176</td>
</tr>
</tbody>
</table>

After verifying that rNoxA-3 could oxidize NADH in the presence of DCPIP at 37 °C and at 60 °C, a kinetic study was performed (Figure 4.4). At 60 °C the $K_m$ for NADH is 4.71 ± 1.3 µM and the $V_{max}$ is 1.6 ± 0.06 U/mg.

![Figure 4.4. Kinetic activity of rNoxA-3 with DCPIP at 60 °C.](image)
Activities of DsrAB from *D. vulgaris* and *A. fulgidus* with the potential electron donors

To study the potential electron donors for the dissimilatory sulfite reductase, biochemical and biophysical techniques were employed. As discussed before, three ferredoxin oxidoreductases (PFOR, AOR and CODH) and one NADH oxidase were chosen. Concerning PFOR, Oliveira *et al.* (2011) already generated a theoretical structure model for the possible interaction between PFOR and DsrAB (*D. vulgaris*). They verified that the surface electrostatic potential for the *D. vulgaris* DsrAB protein surface is negatively charged around the region of the ferredoxin domain (DsrB), which is potentially the region for interaction with the electron donor. In the PFOR analysis, the ferredoxin binding domain is positively charged. Using Pymol as the modeling tool, they verified that the PFOR ferredoxin binding domain can be positioned in the vicinity of the *D. vulgaris* DsrAB ferredoxin domain. This analysis positioned the iron–sulfur clusters of the two domains at ~15 Å from each other, suggesting that electron transfer from the PFOR catalytic center to the DsrAB (*D. vulgaris*) [4Fe-4S] cluster is possible (Oliveira *et al.*, 2011). On the other hand, AOR, CODH and NoxA-3 were not yet analyzed as possible electron donors.

Two approaches were conducted to test these possible electron donors: i) the analysis of the sulfite consumption/product formation by DsrAB (*D. vulgaris* and *A. fulgidus*) in the presence of the different possible physiologic electrons donors in comparison to the DsrAB activity using methyl viologen as the artificial electron donor, and ii) the analysis of protein-protein interactions using Surface plasma resonance (SPR).
Ferredoxin oxidoreductases

To analyze the consumption of sulfite/product formation by DsrAB (D. vulgaris and A. fulgidus) the monobromobimane (mBbr) method was used. In this method, mBbr reacts with free thiols, such as sulfite, sulfide and thiosulfate. The first attempt was to analyze the consumption of sulfite. Unfortunately, it was impossible to extrapolate any conclusion from the sulfite consumption, as the data values for the sulfite consumption during the time period were poorly reproducible (Figure 4.5). The reason for these oscillation in the data could be due to the method of collecting the sample. The enzymatic reactions were performed in a 500 µL tube with 300 µL of total reaction. At each data point the system was opened (inside of the anaerobic chamber) and a sample of 10 µL was taken to analyze by HPLC, which accounted for a loss of 20% of total volume at the end of the experiments. The small reaction volume along with the disturbance of the atmospheric equilibrium (at 37 and 60 °C) might had led to changes in sulfite concentration, which masked the results. Therefore, we analyzed instead the product formation. In these analyzes we were expecting to find production of thiosulfate as it is found when Mv is used. As it can be

![Graph A](image1)

![Graph B](image2)

Figure. 4.5. Analysis of sulfite consumed by DsrAB of A. fulgidus (A) and D. vulgaris (B) in the presence of the possible physiological electron donors. Mv as the positive control and Hase (hydrogenase) alone as a negative control.
verified in the Figure 4.6, when we analyzed thiosulfate production none of the possible physiological electron donors behaves similarly to the artificial electron donor (Mv). Only AOR seemed to support some level of activity.

**Figure. 4.6.** Analysis of thiosulfate production of DsrAB of A. fulgidus (A) and D. vulgaris (B) in the presence of the possible physiological electron donors. Mv as the positive control and Hase (hydrogenase) alone as a negative control.

**NADH oxidase (rNoxA-3)**

To analyze the interaction of rNoxA-3, a different approach was used, as we measured instead the oxidation of NADH at 340 nm with DsrAB and sulfite as electron acceptors (Figure 4.7). However, the number of mols of NADH needed to reduce sulfite is complex as it depends on the products formed (Table 4.4).
Table 4.4. Number of mols of NADH needed per sulfite product.

<table>
<thead>
<tr>
<th>mols NADH</th>
<th>sulfite reduction product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 mol thiosulfate</td>
</tr>
<tr>
<td>2</td>
<td>1 mol trithionate</td>
</tr>
<tr>
<td>3</td>
<td>1 mol sulfide</td>
</tr>
</tbody>
</table>

In many assimilatory sulfite reductases, a NADPH-dependent subunit functions as the electron donor for sulfite reduction (Crane & Getzoff, 1996). Similarly in the SOB A. vinosum, the reverse dissimilatory sulfite reductase is co-purified with DsrL, an NAD(P)H:acceptor oxidoreductase. Also, in E. coli, the cytoplasmic NirB nitrite reductase, of the same siroheme-dependent family as DsrAB, is a NADH-dependent enzyme capable of reducing nitrite to ammonium (Wang & Gunsalus, 2000). These factors suggest a probable interaction of dissimilatory sulfite reductase with an NAD(P)H-dependent oxidoreductase. This would connect sulfite reduction to the regeneration of NAD⁺.

We first checked for the activity of rNoxA-3 at 37 °C and 60 °C, at pH 7 and in Kpi 50 mM buffer following the decrease of the DCPIP absorbance at 600 nm. The activities were 0.101 U/mg at 37 °C and 1.250 U/mg at 60 °C. Inside the anaerobic chamber the electron acceptor DCPIP was substituted by sulfite (0.5 mM for DsrAB from A. fulgidus and 2.5 mM for DsrAB of D. vulgaris) and the DsrAB protein of D. vulgaris (37 °C) and A. fulgidus (60 °C), and the activity as followed by NADH decrease at 340 nm. However, we observe no oxidation of NADH in these conditions, suggesting that rNoxA-3 cannot transfer electros to DsrAB in the conditions of this assay.
Surface plasma resonance (SPR)

To test protein-protein interactions independently from enzymatic assays, SPR was employed. The CM5 chip (amine binding) was selected to bind DsrAB from *D. vulgaris* and *A. fulgidus*. All the proteins previously referred were tested: PFOR, AOR, CODH and rNoxA-3. The SPR corroborated the data obtained by the *in vitro* activities experiments, as no interaction between these potential electron donors and both DsrABs was observed. Having the proteins already bound to the chip we decided to test other possible electrons donors, soluble and membrane proteins, as these proteins were already available in our laboratory. The proteins were: Tmc and Qrc from *D. vulgaris* Hildenborough, Hmc from *D. gigas*, and DsrMKJOP from *D. desulfuricans* ATCC 27774 (membrane complexes), and ferredoxin I from *D. gigas*. No interaction with these proteins was observed for DsrAB from *A. fulgidus*, which presented only a week interaction with the Hmc membrane complex and with ferredoxin I. However, DsrAB from *D. vulgaris* showed some interaction with all membrane complexes and also with ferredoxin I. This difference between DsrAB from *D. vulgaris* and *A. fulgidus* is probably due to the fact that the *D. vulgaris* protein has DsrC bound to it. It was already proven by Venceslau et al. (Venceslau, 2011) that DsrC is able to bind to DsrMKJOP, and this shows that DsrC can interact with the membrane complex even when bound to DsrAB.
Genomic analysis of ferredoxin-like proteins in SPR

As referred before Larsen et al. did a multiple alignment analysis where they grouped together a ferredoxin (Fdx) from *D. thermocisternum* (immediately upstream of dsrAB genes) along with the Fdx-4 of *A. fulgidus* and Fdx-I from *D. vulgaris* (Larsen et al., 1999). We decided to extend this analysis for all the available genomes present in the list for sulfate reducers at IMG. As so, we compared the Fdx-4 from *A. fulgidus* (AF0427) against the SRP list. From this comparison, forty seven genomes had at least one Fdx homologue to Fdx-4 of *A. fulgidus*. Moreover, thirteen Fdx homologues were found nearby a dsr gene (Figure 4.8). From this analysis, the thirteen organisms can be divided in two main groups: the Gram-negative SRP (Figure 4.8 A) and Gram-positive SRP (Figure 4.8 B). In both cases the fdx gene is near the dsrAB-D genes or dsrMKJOP/dsrMK-C genes. This results supports the proposal that this ferredoxin protein can act as the possible physiological electron donor.

In our *in vitro* experiments we tried to use the ferredoxin (Fdx) from *Clostridium tetanomorphum*, as an intermediate between AOR and DsrAB. This experiment failed to prove the interaction between DsrAB and the Fdx. Nevertheless, this Fdx may not be the best candidate for DsrAB interaction, as it has a molecular weight of ~26.7 kDa, while the ferredoxins near the dsr genes are smaller, ~6.6 kDa. The difference in size and the fact that the ferredoxin and the DsrAB used are from different organisms, probably influenced the interaction with the ferredoxin domain in the vicinity of the siroheme. The next step is to purify the ~6.6 kDa ferredoxin from *D. vulgaris* or *A. fulgidus* and evaluate their interaction.
Figure 4.8. Neighborhood organization of the genes homologous to fdx-4 gene from A. fulgidus (AF0427). (A) Gram-negative SRP, (B) Gram-positive SRP. In the green box are represented the homologues to the Fdx-4. Red, orange and blue boxes identify the dsrAB-D, dsrMK-C and dsrMKJOP genes respectively (This scheme was obtain using the IMG database website)
4.4. Conclusion

According to our experiments the ferredoxin oxidoreductases PFOR, AOR and CODH were not capable of sustaining DsrAB activity (*D. vulgaris* and *A. fulgidus*). Also, the NADH oxidase rNoxA-3 was not able to interact with DsrAB to reduce sulfite to sulfide. We also tried to use the ferredoxin (from *C. tetanomorphum*) as an intermediate between the AOR and DsrAB, also with negative results. These results do not mean that these candidates are not physiological partners, only that in the conditions in which the experiments were performed they did not succeed. In our perspective, ferredoxin is still a good candidate to be involved with DsrAB for sulfite reduction due to the fact that at least in thirteen genomes, of the total forty seven genomes, a homologue of Fdx-4 is found near *dsr* genes. The small ferredoxin from *A. fulgidus* (AF0427) would be a good candidate for heterologous expression and to test as the electron donor for DsrAB proteins. Further work needs to be performed in order to find the physiological electron donor for the DsrAB dissimilatory sulfite reductase.
Searching for the DsrAB physiological electron donor

Chapter 4

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Searching for the DsrAB physiological electron donor


Chapter 5

DsrJ, the cytochrome c subunit of the DsrMKJOP membrane complex
Chapter 4

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5.1. Introduction

Membrane respiratory complexes are of extreme importance for life as they are the link to energy conservation in living organisms. There are two essential membrane complexes for sulfate reduction present in all SRP and also some SOB: DsrmKJOP and QmoABC (Frigaard & Dahl, 2009, Pereira et al., 2011). The QmoABC complex is thought to exchange electrons with the ApsBA (APS reductase) and DsrmKJOP is proposed to reduce the trisulfide form of DsrC. The DsrmKJOP membrane complex was first purified from A. fulgidus in 2002 by Mander and coworkers (Mander et al., 2002), where it was named Hme forHdr-like menaquinone-oxidizing enzyme (Hme). A homologue to Hme complex was also discovered in A. vinosum by Dahl et al (Dahl et al., 2005). It was found to be part of an essential operon for sulfur oxidation coupled with the dsrAB genes, and for this reason was named DsrmKJOP complex. In 2006, Pires et al purified the DsrmKJOP complex from D. desulfuricans ATCC 27774 which was shown to be a homologue of the Hme complex of A. fulgidus (Pires et al., 2006). This complex is formed by five subunits. One cytoplasmic subunit, DsrK, thought to be the catalytic subunit of DsrmKJOP complex due to its homology to HdrD, which is responsible for heterodisulfide reduction by the membrane-bound HdrDE complex. Two membrane bound subunits (DsrM and DsrP), possibly responsible for menaquinol interaction, and two periplasmic facing membrane bound proteins (DsrO and DsrJ) (Pires et al., 2006, Grein, 2010). DsrJ contains three heme binding motives CXXCH, in which one of these hemes is characterized by an unusual His/Cys heme distal axial ligation (Pires et al., 2006, Grein et al., 2010). In the DsrmKJOP from D. desulfuricans a redox potential of - 400 mV and a very unique peak $g_{max}$ 2.47 for the EPR spectra were attributed to this His/Cys heme ligation (Pires et al., 2006). This very low redox
potential was also found in SoxXA from *Rhodovulum sulfidophilum*. SoxXA are heme-containing proteins crucial for the initiation of thiosulfate oxidation (Cheesman *et al.*, 2001). Also, EPR spectra of SoxXA showed a low-spin heme signal in the range of $g_{\text{max}} = 2.5$, characteristic for the His/Cys coordinated heme groups (Walker, 1999, Cheesman *et al.*, 2001, Kappler *et al.*, 2005). Although unusual, this heme cysteine ligation was proven not to be essential for SoxXA reaction mechanism, as the SoxXA cysteine mutant (change by a methionine) could maintain 55% of the wild type SoxXA glutathione-based activity (Kilmartin *et al.*, 2011). Also, in the mutated *A. vinosum* DsrJ strain, where the cysteine was substituted by a serine, albeit with a significantly reduced oxidation rate, the cells were still capable of oxidizing sulfur (Grein *et al.*, 2010). Nevertheless, in both SoxXA and DsrJ cysteine mutants, their activity decreased significantly pointing out that, although not crucial, these His/Cys heme ligations are extremely important for the overall function of these proteins (Grein *et al.*, 2010, Kilmartin *et al.*, 2011). Moreover, the crystallographic structure of DsrJ is still not available, which would provide the information needed to address the question of which amino acids are the distal axial ligands for the three hemes.

The work described aimed to disclose the true identity of all the ligands to the three heme of DsrJ. To accomplish this, mutants in the conserved residues that could be the distal axial heme ligands were performed, and were then analyzed by Electron paramagnetic resonance, Raman Resonance, UV-visible and Mass spectrometry.
5.2. Methods

Competent cell transformations

Plasmid DNA was inserted into *E. coli* strains by transforming chemically competent cells. Per each transformation, 1 µl of plasmid DNA or 5 µl of a ligation assay were added to 50 µl of competent cells. The preparation was first incubated for 60 min on ice followed by 90 s at 42 °C and 2 min on ice. These competent cells were then added to 500 µl of LB medium and incubated at 37 °C for 60 min with agitation. Finally, different aliquots of the assay were applied onto LB plates containing the respective antibiotic.

Sequence and ligation independent cloning (SLIC)

To produce the variants, the selected plasmid (pETJStrep or pETStrepJ) was amplified by PCR in two parts that carried the desired mutation at the (compatible) end of the fragments. Fragments were amplified using the HercII polymerase and using the following primer pairs: fragment A (forward primer introducing the mutation and reverse primer for the ampicilin gene) and fragment B (forward Ampicilin primer and reverse primer introducing mutation). The role of cysteine 46 was already studied before by replacing this amino acid by a serine (Grein, 2010, Grein *et al.*, 2010). Since it could not be excluded that serine can ligate a heme iron, the cysteine was here replaced by a glycine. The methionines M53, M58 and the histidine H57 were replaced by alanines. All possible combinations of mutants were created generating 10 different constructions. To create the respective mutation the entire plasmid was PCR amplified in two parts, where both parts carry a short overlapping sequence which carries the desired mutation. The parts
Chapter 4

were then fused using the SLIC method (Li & Elledge, 2007). This protocol was used to insert the single point mutations. First a PCR of the plasmid fragment(s) with HercII polymerase was performed. After cleaning the PCR products with the Fermentas kit (GeneJET Gel Extraction Kit), the products were quantified using Nanodrop and their weight verified on an agarose gel. The plasmid fragments were then mixed in a tube so that there were in equal molar ratios of each and sterile water was added to make a total volume of 15 µl. To this volume, 5 µl of T4 prep mix (6 µl BSA (1 mg/ml), 6 µl NEB Buffer 2 (10x), 2.5 µl sterile deionized water and 0.5 µl T4 DNA polymerase (3 U/µl)) were added. This mix was incubate at room temperature for 30 min, after which 2 µl of dCTP (10 mM) were added. To allow proper annealing of the complement single stranded regions this final mix was incubated at 37 °C for 20 min. Finally 5 µl of the SLIC mix were transformed into E. coli DH5α.

DsrJ expression conditions

The expression of DsrJ from A. vinosum performed was described in (Grein et al., 2010). Briefly, E. coli BL21 (DE3) was first transformed with the pEC86 plasmid to provide the expression of the genes encoded in the ccm operon under aerobic conditions (Arslan et al., 1998), and was transformed with the appropriate plasmid used for expression of dsrJ gene. The fully heme-loaded DsrJ holoprotein was obtain by slow production of the recombinant protein in E. coli. The lacT7 promoter was used to allow the minimum expression level in the pET22b vector. No induction by IPTG was performed. 800 mL of NZCYM medium in a non-baffled Erlenmeyer flask were inoculated with a single colony of the appropriate clone or directly from a glycerol stock culture and grown at 37 °C with 150 rpm agitation for 16 hours.
**DsrJ purification**

*Affinity tag purification.* The purification of DsrJ was accomplished by affinity chromatography. In this purification buffer W (150 mM NaCl, 100 mM TrisHCl pH 8) was used to equilibrate the column. Cells from 800 mL were broken in a French Press equipment and centrifuged at 58545 × g for 20 min. The supernatant was then added to the Streptactin column and purified by gravity. After all the sample was applied to the column, 5 column volumes (CV) of buffer W were passed. Finally, to elute the protein from the column seven half CV of buffer E (150 mM NaCl, 100 mM TrisHCl pH 8 and 2.5 mM desthiobiotin) were passed. The column was regenerated with one CV of 0.5 M NaOH and 10 CV of buffer W.

*Purification of the rDsrJ without the strep tag.* The cells were broken with the French press, centrifuge 15 min at 58000 × g and the supernatant collected and centrifuge at 100000 × g for 1:30 h. The soluble fraction was applied in a Q-Sepharose 16/10 with and without detergent (SB12). The fraction with the highest 280/408 nm was applied into resource 6mL. The best fraction from the last column was applied to an HTP column.

**Protein detection**

For the detection of the DsrJ protein a 9% tricine-SDS-PAGE was performed was described in (Schagger, 2006). The gel were electrophoresed for 2 h at 120 mV in an Amersham electrophoresis equipment. To reveal the protein bands Coomassie and heme staining were employed.
Coomassie staining. After protein electrophoresis the stacking gel was removed, and the resolving gel was transferred into a staining solution containing 50% (v/v) methanol, 10% (v/v) acetic acid, 40% (v/v) dH₂O and 0.25% (w/v) Coomassie Brilliant Blue R250, during 30 min. Next, the staining solution was replaced by a destain solution with 20% (v/v) methanol, 10% (v/v) acetic acid and 70 % (v/v) dH₂O. The latter was exchanged several times, until the protein bands were clearly visible.

Heme staining. After the polyacrylamide gel electrophoresis the stacking gel was removed and the resolving gel incubated in the heme staining solution (4.5 mg of TMBZ dissolved in 15 mL methanol with 35 mL of 250 mM sodium acetate pH 5) for 30 min in the dark with gentle agitation. Then an incubation with 600 μl of a 30% H₂O₂ solution was added and the gel left in the solution until signals appeared. The reaction was stopped by several repeated wash steps with MilliQ water.

Mass spectrometry

The mass spectrometry measurements were carried at the Mass Spectrometry Laboratory, Analytical Services Unit, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa. For each sample 5 μl of protein were desalted, concentrated and eluted using R1 (RP-C4 equivalent) microcolumns. Proteins were eluted directly onto a MALDI plate with sinapinic acid (10 mg/mL) using 50% (v/v) acetonitrile and 5% (v/v) formic acid. Mass spectra were acquired in the positive linear MS mode using a MALDI-TOF/TOF, Applied Biosystems, model 4800 (PO 01MS).
**EPR spectroscopy**

Electron paramagnetic resonance (EPR) spectroscopy was accomplished by Ines A. C. Pereira and Sofia S. Venceslau from the Laboratory for Bacterial Energy Metabolism of the Instituto de Tecnologia Quimica e Biologica (ITQB), Universidade Nova de Lisboa. Spectra were recorded using a Brucker EMX spectrometer equipped with an ESR 900 continuous-flow helium cryostat from Oxford Instruments.

**Raman Resonance**

RR spectra were recorded by Smilja Todorovic in the Laboratory for Raman Spectroscopy of Metalloproteins at the ITQB. Measurements were performed with a confocal microscope coupled to a Raman spectrometer (Jobin Yvon U1000). Samples were placed in a quartz rotating cell, excited with either 413 nm or 647 nm line from a krypton ion laser (Coherent Innova 302) and measured with 5 mW laser power and accumulation times of 60 s at room temperature. After polynomial background subtraction, the positions and line-widths of the Raman bands were determined by component analysis (Todorovic et al., 2006).

**NMR experiments**

NMR experiments were performed by Catarina Paquete from the Inorganic Biochemistry and NMR Laboratory at the ITQB. The buffer of the purified proteins was exchanged for 80 mM sodium phosphate buffer (pH 8.0) with NaCl (final ionic strength of 250 mM) prepared in 99.9% $^2$H$_2$O (CIL), through ultrafiltration procedures with Amicon Ultra Centrifugal Filter Units (Millipore). Protein samples with
approximately 1 mM were placed in 3 mm Wilmad NMR tubes. The 1D $^1$H NMR spectra were acquired on a Bruker Avance 600 MHz spectrometer with a spectral width of 30 kHz at 25 °C. $^1$H chemical shifts were calibrated using the water signal as internal reference. All NMR spectra were processed using TopSpin™ NMR Software from Bruker Biospin.

### 5.3. Results and discussion

DsrJ is part of a larger membrane complex believed to be involved in sulfate respiration. This protein was already investigated by Pires et al. as part of the DsrMKJOP complex from *D. desulfuricans* (Pires et al., 2006). Grein et al. had also examined the role of DsrJ in the DsrMKJOP complex of *A. vinosum* (Grein et al., 2010). However, the nature and function of this cytochrome c was not completely revealed. DsrJ contains, by sequence analysis and confirmed by mass spectrometry, three c-type hemes. According to sequence alignment only one cysteine,

![Figure 5.1](image)

*Figure. 5.1. Sequence alignment of DsrJ proteins. Heme binding motifs are boxed. Putative distal heme ligands are denoted with arrows. Strictly conserved residues are marked with black boxes. Species: A. vinosum (Avin), Magnetococcus sp. MC-1 (McMC-1), Thiobacillus denitrificans (Tden), Chlorobaculum tepidum (Ctep), Desulfovibrio vulgaris Hildenborough (Dvul), Desulfovibrio desulfuricans ATCC 27774 (Ddes), Archaeoglobus fulgidus (Aful), Desulfomicrobium baculatum (Dmbac), and Candidatus Ruthia magnifica (CandRmag) (Grein et al., 2010).*
two methionines, one histidine, one serine, one arginine and one phenylalanine are conserved among DsrJ homologue sequences (Pires et al., 2006, Grein et al., 2010).

Considering that there are no documented cytochrome c-type hemes with a serine or phenylalanine as distal axial ligands, these were not considered as possible ligands. Also, in SoxXA (present in SOB and involved in thiosulfate oxidation) the conserved arginine is proposed to be responsible for the sulfur-sulfur bond polarization, providing a strong anion-binding site suitable for the substrate, thiosulfate (Bamford et al., 2002). As so, only the remaining residues were considered as probable heme ligands, one cysteine, two methionines, and one histidine. Pires et al proposed that, the hemes have His/His, His/Met and a unique His/Cys coordination, based on sequence analysis and EPR spectroscopy. This His/Cys ligation is so unique that it has only been reported in three other proteins: SoxXA, a complex involved in thiosulfate oxidation, isolated for example from Rhodovulum sulfidophilum (Reijerse et al., 2007), PufC; also from this organism, a protein associated with the photosynthetic reaction center (Alric et al., 2004); and TsdA, a thiosulfate dehydrogenase (Denkmann et al., 2012). In A. vinosum it was proven that DsrJ is essential for sulfur oxidation (Sander et al., 2006), and it was also verified that a mutant where the conserved cysteine was changed to a serine had a severe decrease in the rate of sulfur oxidation, retaining only 19% of the wild type activity (Grein et al., 2010). Although these distal axial heme ligands were assigned to DsrJ, the heme coordination was never completely established. The only evidence is for the His/Cys heme was a characteristic peak at $g_{\text{max}} = 2.47$ observed by EPR. Besides the EPR, it was not yet possible to assign the His/Cys heme-coordination by other technique. For this reason, we continued the work performed by Grein and coworkers (Grein et al., 2010), using their vector we generate
several variants of DsrJ with mutations in the putative heme ligands to try to establish the heme coordination. According to Grein et al. (Grein et al., 2010), the vector pETJStrep introduced in *E. coli* BL21 (DE3) gold, is able to produce a full heme loaded protein. As so, we used the same growth conditions during this work. Several approaches were used to express rDsrJ in *E. coli*, which involved the generation of the variants using different constructions: i) the rDsrJ with the Strep tag in the N-terminal, ii) rDsrJ with the Strep tag in the C-terminal position and iii) rDsrJ without the Strep tag.

**rDsrJ with the Strep tag in the C-terminal**

5’- **MKYLLPTAAAGLLLLAAQPAMAMDEVKRYVVEGSPAERE**SCVEPTETMRRMHMEFIKHQRISTVHEGIRGTKYSLTGCVDCHISYDANRNPPQPI
DQPĐQFCGACHNYAAVDLNCFDCHASVNPRTGADADAEEAHRAAGVTGAPHGGAHSAWSHPQFEK -3’

*Figure 5.2. Sequence organization with the pELB leader (blue), strep tag (green) and DsrJ gene (red).*

Our first approach was to use the plasmid pETJStrep (Grein et al., 2010), which is a pet22b vector in which the His tag was replaced by a pPR-IBA2 Strep tag in the C-terminal (organization in the Figure 5.2) (Grein et al., 2010). This vector contains the DsrJ gene (Alvin_1260) from *A. vinosum* without the first 26 amino acids present in the N-terminal that code for a signal peptide required for the translocation of the protein via the Sec pathway. This signal peptide was removed because the plasmid already encodes an equivalent leader peptide. With this plasmid, seven variants were generated: C46G, M53A, M58A, C46GM53A, C46GM58A, M53AM58A and C46GM53AM58A.
**UV-visible spectroscopic analysis.** The UV-visible spectrum was similar for all the rDsrJ variants. It shows the characteristic peaks of a cytochrome c with the Soret peak at 408 nm and a δ peak at 351 nm in the oxidized state and a Soret peak at 417 nm and α and β peaks at 520 nm and 550 nm respectively in the reduced state (Figure 5.3). Also, a band at 650 nm appears both in the oxidized and reduced state. This band was analyzed by Grein et al., with resonance Raman, and it was associated with an electronic absorption band belonging to a Q-band (α/β transitions) (Grein et al., 2010). The 650 nm peak is similarly detected in the spectrum of the ferric P450 cytochromes in which a Cys is a proximal ligand (Yoshioka et al., 2001), in some substrate-bound LS P450 cytochromes and in Cys/His P450 mutants (Martinis et al., 1996). It has also been correlated to a five-coordinated high-spin species. Due to the fact that this 650 nm band was observed in the rDsrJ Wt (which according to Grein and coworkers there was no high spin observed) and in all the variants, we did not assign this 650 nm to the presence of a high spin heme.

![UV-visible spectrum of rDsrJ wild type](image)

**Figure. 5.3.** UV-visible spectrum of rDsrJ wild type in the oxidized state (black) and in the reduced state (red). The box on the right is a close up to α and β peaks and also to the 650nm shoulder.
**Mass spectrometry analysis.** According to the MS analysis the rDsrJ Wt, the rDsrJ C46G and the rDsrJ M53A all had approximately the correct molecular mass concerning the native form along with 3 hemes (Table 5.1). This suggests that the maturation time and the purification of the proteins were well established.

![Table 5.1. Mass (Da) obtained versus the theoretical mass for the variants produced and the number of hemes present per each protein. MD (Mass difference between deviation and theoretical mass of the 3 hemes (1837.5 Da)).](image)

<table>
<thead>
<tr>
<th></th>
<th>theoretical</th>
<th>obtained</th>
<th>deviation</th>
<th>theoretical mass of heme c</th>
<th>MD</th>
</tr>
</thead>
<tbody>
<tr>
<td>rDsrJ wild type</td>
<td>14960.5</td>
<td>16812.2</td>
<td>1857.7</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>rDsrJ C46G</td>
<td>14914.4</td>
<td>16760.9</td>
<td>1846.5</td>
<td>612.5</td>
<td>9</td>
</tr>
<tr>
<td>rDsrJ M53A</td>
<td>14900.4</td>
<td>16760.7</td>
<td>1860.3</td>
<td></td>
<td>23</td>
</tr>
</tbody>
</table>
We also perform a more thorough analysis of the MS spectra of rDsrJ wild type and rDsrJ C46G. Comparing our results with data from Grein et al. 2009 (unpublished data), together these MS analyses indicate that there might be a modification in the wild type protein (Figure 5.4). The rDsrJ Wt protein and the rDsrJ M53A shows an additional peak that varies between 104 and 115 Dalton, besides the expected peak for DsrJ with 3 hemes. This peak is clearly missing in the C46S and C46G variants. Due to the proximity of the mass variation of this additional peak to the mass of a thiosulfate molecule (112 Da), we propose that, both rDsrJ Wt and the rDsrJ M53A, may have a thiosulfate modification of one heme. A similar modification is also observed in the heme-ligating cysteine of SoxXA, which is in a persulfide form, since the mass increased by 32 Da (Bamford et al., 2002). The origin of this modification is still not clear. The persulfide form was observed in
Rv. sulfidophilum SoxXA as result of the turnover of the protein or incomplete reaction cycles and when the SoxXA was incubated with thiosulfate, dimethylsulfide (DMS) or dimethylsulfoxide (DMSO) it also give rise to additional peak increase (Kappler et al., 2005).

**Raman Resonance (RR) analysis.** To obtain information on the redox state, spin state and ligation pattern of the hemes with RR the spectra must be at the high frequency region (1300 – 1700 cm⁻¹). In this region the spectra of the proteins are obtained upon Soret band excitation. The high frequency RR spectra were therefore recorded upon Soret band excitation at 413 nm (Figure 5.5). The ν₄ band is a marker band for the redox state of the heme iron whereas ν₃, ν₂ and ν₁₀ bands are sensitive indicators of the heme spin state (Spiro & Czernuszewicz, 1995). The RR spectra of all mutants revealed a ν₄ band centered at ~1373cm⁻¹ typical for ferric cytochrome c (Spiro & Strekas, 1974). The Raman resonance (RR) spectra of the DsrJ variant proteins are extremely similar to each other. Even the proteins missing the cysteine 46 and methionine 53 show identical spectra as the wild type protein. This was also observed for the C46S protein (Grein et al., 2010). Nevertheless RR spectroscopy confirms that there is no high spin heme in any of the samples (Figure 5.5).
Figure. 5.5. Overlaid resonance Raman spectra overlaid of all variants analyzed, proving the absence of high spin heme in any sample.
**Electron paramagnetic resonance (EPR).** All DsrJ variant proteins were analyzed by EPR spectroscopy (Figure 5.6). EPR spectra also did not reveal the presence of high spin hemes. The $g_{\text{max}} = 2.517$ signal which was attributed to a thiolate ligated heme disappears either when C46 or M53 are missing in the protein.

*Figure. 5.6. EPR spectra of all mutants analyzed comparison. No signal is visible for a high spin heme. The only difference is the shoulder at $g_{\text{max}}=2.517$ (red line) that disappears in variants where the C46 or the M53 were modified. Experimental conditions: microwave frequency, 9.38 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; and modulation amplitude, 1 mT*
rDsrJ with the strep tag in the N-terminal

5'- MKYLLPTAAAGLLLLAAQPAMA MASWSHPQFEK GAGSDEVKRYVVE GSPAARESCVEPTETMRRMHEFIKHQRISTVHEGIRGTKYSLTGCVD CHISYDANRNPQPIDQPDQFCGACHNYAAVDLNCFDCHASVPNRPGAD AEAHRAAGVTGAPHGGGH- 3'

Figure 5.7. Sequence organization with the pELB leader (blue), strep tag (green) and DsrJ gene (red)

During the first approach we realized that the proteins were being purified together with a second form due to the proteolytic degradation of rDsrJ (Figure 5.8 B). Since this form also stains for heme, it will distort the spectroscopic characteristics of the sample and also the protein concentration calculation. Even though this form could be removed with a S75 gel filtration column, it was decided to change the strep tag from the C-terminal to the N-terminal. In this new strategy if the proteolytic degradation of rDsrJ that binds to the Streptavidin column it will not interfere with the heme calculations as it does not contain the heme binding motif. This new approach improved the purification step without the interfering of the proteolytic degradation in the heme analysis (Figure 5.7). This new construction was named petStrepDsrJav (rDsrJ2wt), and from it seven variants were generated: C46G, H56A, C46GM53A, C46GM58A, C46GH56A and M53AM58A. Once again, there was no change in the UV-visible spectra of the variants. Intriguingly, not even the H56A variant had extra peak for a high spin heme, which is quite strange as this is the only histidine conserved among all DsrJ proteins and it should be one of the heme-ligands in this protein.

Figure 5.8. Heme staining of rDsrJ wild type (A) and the proteolytic form (B)
**Nuclear magnetic resonance (NMR).** NMR is an extremely good technique to study heme ligations and heme environment changes. The samples rDsrJ2wt and the variant rDsrJ2C46G were purified and analyzed by NMR (by Doctor Catarina Paquete from Inorganic Biochemistry and NMR Laboratory at ITQB-UNL Portugal). When comparing rDsrJ2wt with rDsrJ2C46G there is no peak differences from these two samples. However, the absence of the cysteine may be affecting the heme environment. When the cysteine is modified to a glycine, in the NMR spectrum we can verify the appearance of a shoulder in the main peaks of the rDsrJ2C46G variant (Figure 5.9). According to the temperature assay (Figure 5.9), it can be observed that both samples start with the same peaks at the same ppm, with increasing temperature it can be noticed that in both cases there is a peak shift. However, in the case where the cysteine is absent (rDsrJ2C46G variant), a shoulder starts to appear (with increasing temperature) in the peaks at ~35 ppm and ~21 ppm (Figure 5.8 blue arrows). This may suggest a change in the heme environment.
Figure 5.9. Temperature dependence of the samples (A) rDsrJ2wt and (B) rDsrJ2C46G. The arrows in (B) indicate the appearance of the shoulders.
**rDsrJ without the Strep tag**

Due to the ambiguous results described above, concerning the heme ligands we thought that the strep tag could be influencing the overall structure of rDsrJ. To remove the tag we used two approaches: to simply remove it from the vector or to use a 6His-tag instead with a thrombin cutting sequence in the N-terminal to enable its removal.

The recombinant protein without any tag revealed to be extremely difficult to purify. Although three columns were performed, the pure protein had a very low 408/280 nm ratio when comparing with what is obtained when purifying the rDsrJ protein with the strep tag. This ratio was no higher than 0.26 in comparison with 5 from the strep tagged protein. Another drawback of this new approach was the fact that rDsrJ was spread through several peaks. From this strategy it was never possible to obtain pure protein to work with.

Our last approach was to introduce the *dsrJ* gene without the first 26 amino acids into pet28c (Figure 5.10). This new pET vector contains a 6His-tag in the N-terminal position followed by a thrombin cutting sequence. The optimization of this method is still ongoing.

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5’- MKYLPTAAAGL.LAAQPAMA MGSSHHHHHSGLVPRGHMDE  
VKRYVEGSPAERESCVEPTETMRRMHEFIKHQRISTVHEGIRGTK  
YSLTGCVDCHISYDANTNPQPIDQPDCFGACHNYAAVDLNCFDCHA  
SVPNRPGADAEAAHRAAGVTGAPHGGG -3’
```

*Figure. 5.10. Sequence organization with the pELB leader (blue), 6his tag (green), thrombin cutting site (yellow) and DsrJ gene (red)*
5.4. Conclusion

DsrJ is a very special $c$-type cytochrome. Although it has been studied since 2006 (Pires et al., 2006, Grein et al., 2010) the heme ligands still remain to be clearly established. Here, the continuation of the work performed by Grein and coworkers was described. We performed more than ten variants and analyzed the recombinant proteins by several techniques such as EPR, RR, NMR and UV-visible. The results were not elucidative as they do not corroborate the previous results on heme ligands assumptions (Pires et al., 2006). It was predicted by Pires that the conserved cysteine 46, the histidine 57 and the methionine 58 should be the three distal axial ligands of DsrJ hemes. These proposals could not be supported by the variants performed, including the cysteine and the conserved methionines, as they did not reveal any high spin heme in any of the techniques used. This may mean that either there are other residues (not conserved among different DsrJ proteins) that can ligate the hemes in the absence of the preferred distal axial ligand (the conserve residues) predicting a “switch on/off” mechanism, or simply that something is interfering with the heme coordination, and so these results are not conclusive. Furthermore, we cannot exclude the possibility of a change in the native structure of DsrJ, due to the absence of the other subunits of the DsrMKJOP complex. Attempts to crystalize DsrJ failed, as none of the variants produced crystals. The structure of DsrJ could close the discussion, as it would answer the question of the heme ligands.
References


Chapter 4


Chapter 6

Concluding remarks
Concluding remarks

Over the decades the mechanism of sulfate respiration has been uncovered. One of the main questions was the mechanism of the six electron reduction of sulfite to sulfide by DsrAB. For several years, the mechanism proposed was based in *in vitro* assays, in which DsrAB produces three products, in relative proportions that depend on the sulfite concentration available. The enzyme produces more trithionate/thiosulfate in the presence of high concentrations of sulfite, or more sulfide in the presence of lower concentrations of sulfite. However, in 2008 Oliveira and coworkers suggested that DsrC could be crucial for this mechanism. With this, Oliveira and coworkers proposed a different mechanism for sulfite reduction based on the crystal structure of DsrAB from *D. vulgaris* Hildenborough. In this mechanism, the C-terminal conserved cysteines (Cys93 or Cys$_B$ and Cys104 or Cys$_A$) of DsrC would be essential for the catalytic reduction of sulfite. According to this proposal, DsrAB would reduce sulfite to an $S^0$ intermediate state at the siroheme-[4Fe-4S] catalytic center, then DsrC Cys$_A$ would interact with the $S^0$ intermediate and form a persulfide DsrC-Cys$_A$-SH intermediate. This persulfide DsrC form would be attacked by Cys$_B$ releasing sulfide and forming a disulfide bond between Cys$_B$ and Cys$_A$. Finally, it was proposed that this oxidized form of DsrC would then be reduced by the DsrMKJOP complex. This was the first proposal for a connection of sulfite reduction to energy conservation. Our new findings showed that this proposal was correct in the fact that DsrC is crucial for the DsrAB sulfite reduction, however DsrC does not form a persulfide but a trisulfide product. We now propose that four electrons from the reduced menaquinone pool will be needed for reducing this DsrC form, instead of the two proposed by Oliveira et al. (2008). According to our data, DsrAB
reduces sulfite to an S\textsuperscript{II} intermediate which is attacked by Cys\textsubscript{A} of DsrC and subsequently by Cys\textsubscript{B} to form a trisulfide bound Cys\textsubscript{B} - S\textsuperscript{0} - Cys\textsubscript{A}. This DsrC-trisulfide will be then reduced at the DsrK heterodisulfide reductase-like protein (present in the DsrMKJOP) by four electrons. Thus, the soluble process accomplished by DsrAB and DsrC is coupled to energy conservation through the generation of a pH gradient driven by the DsrMKJOP. It is thought that DsrK may be able to reduce disulfide bridges and an interaction between the DsrMKJOP complex and reduced DsrC has already been shown. However, this proposal must be validated experimentally, and the redox potential of the DsrC-trisulfide should also be determined.

Furthermore, the source of the first two electrons going into DsrAB and sulfite still needs additional investigation. Based on the structure of DsrAB, which contains one ferredoxin like domain per each DsrA and DsrB subunit, we considered that DsrAB could interact with a ferredoxin oxidoreductase-like protein. Also, in the case of some aSiR and in the reverse DsrAB, NAD(P)H oxidoreductases are crucial as electron donor/acceptor. As such, three ferredoxin oxidoreductases (AOR, PFOR and CODH) and one NADH oxidase were selected as physiologic candidates to test their ability to donate electrons for DsrAB sulfite reduction. Using \textit{in vitro} activity assays and SPR we could not observe any interaction of DsrAB with the selected electron donors. However, these are still preliminary results and further work needs to be performed in order to confirm them. Besides the need to fine tune individual reaction conditions (such as pH and buffer), the next step would be to isolate the electron donor candidates from the same organism as the DsrAB protein. Moreover, there is still the possibility that the electron donor is a ferredoxin protein similar to ferredoxin I of \textit{D. vulgaris} (known to be present in all SRP), as in plants where the aSiR receives electrons from
a ferredoxin previously reduced by the photosystem I. The production of the recombinant 6 kDa ferredoxin from *A. fulgidus* (Fdx IV) would be of extreme importance to test in the activity assays of DsrAB and DsrC.

As referred before, the DsrC is proposed to interact with the DsrMKJOP complex for the reduction of the trisulfide bond after reacting with DsrAB S" intermediate. This complex is widespread among SRP and can be divided in two modules: DsrMK (known to be present in all SRP) and DsrJOP. The DsrMK module is proposed to interact with the menaquinone pool (through DsrM) and to reduce the DsrC-trisulfide bond (through DsrK). On the other hand, the DsrJOP module is very intriguing. It was shown that a mutant lacking the DsrJ subunit was incapable of oxidizing sulfur globules in SOB, although it also exists in SRP, where no sulfur chemistry is supposed to occur in the periplasm. DsrJ is a cytochrome c protein with three heme binding motifs with one proposed to have an unusual cysteine/histidine ligation. To reveal the nature of the heme ligands of DsrJ we generated several variants. These variants were characterized by several techniques: EPR, RR, MNR and UV-visible. Even though we generated more than ten variants, and tested them using different approaches, all the results were inconclusive and did not clarify the nature of the heme ligands. These results suggest that probably something occurred to the recombinant protein masking the results. It could also be that the presence of the other subunits of DsrMKJOP could be extremely important for the proper folding of DsrJ. Moreover, we tried also several attempts to get a crystal structure but none of the variants formed crystals. The crystal structure of DsrJ will be important to solve the question of which are the three distal axial heme ligands. Also, the construction of a *dsrJ* deletion mutant in *D. vulgaris* could answer the question of how important this protein is in SRP.
Overall, this thesis contributed to better understand the role of DsrAB and DsrC in the mechanism of dissimilatory sulfite reduction. It is known that the predominant menaquinone in *Desulfovibrio* (MK-6) has a redox potential of ~-67 mV, while inorganic polysulfides have redox potentials in the range of ~-260 mV. So, the DsrC-trisulfide may have a redox potential too low to be reduced directly by MK-6, suggesting that a quinone bifurcating mechanism may occur in DsrMKJOP. Thus, disclosing the physiological electron donor to DsrAB and the interaction of the DsrC-trisulfide with the DsrMKJOP complex are mandatory to fully understand the sulfite reduction mechanism.