



Fabiana Raquel Silva Oliveira

Bachelor in Biology

**Unravelling the catalytic mechanism for NO reduction
by a flavodiiron protein from *Escherichia coli***

Dissertation to obtain the Master's degree in Biochemistry for Health

Supervisor: Prof. Dr. Miguel Teixeira, Full Professor, ITQB-NOVA

Co-supervisor: Dr. Filipe Folgosa, Investigator, ITQB-NOVA

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Agradecimentos

Várias pessoas foram indispensáveis para a realização do trabalho aqui desenvolvido.

Primeiramente, gostaria de agradecer ao Professor Miguel Teixeira pela disponibilidade e oportunidade de iniciar o trabalho de investigação no seu laboratório. Agradeço também toda a partilha de conhecimento e discussão do meu trabalho ao longo deste percurso. Ao Dr. Filipe Folgosa um especial agradecimento pela partilha de conhecimento científico, por toda a motivação e apoio nos momentos de dúvida, mas principalmente por servir como exemplo de entrega, profissionalismo e sempre com boa disposição, até nos momentos de “Aí que susto!”.

Para além disso, quero agradecer a todos os meus colegas Maria Martins, Susana Fernandes, Silviya Stateva do Functional Biomechistry of Metalloenzymes Lab, por facilitarem a minha adaptação a esta fase académica e por todo o apoio científico e pessoal que deram ao longo deste percurso.

Ao ITQB um agradecimento especial por me ter proporcionado um excelente local de trabalho.

Não poderia deixar de agradecer às minhas amigas Carol, M. Carvalheira, M. Figueiredo, Sofs e Tati, apesar de estar longe estamos sempre perto, sempre com a mesma preocupação, apoio e incentivo para chegar longe e vingar na vida, segue o lema “o que Aveiro uniu ninguém separa!”.

Um especial agradecimento à minha amiga Carol, não há palavras para descrever o tanto que contribuíste para esta tese, direta e indiretamente. Obrigada por toda a paciência e dedicação que sempre tiveste para comigo.

Às minhas amigas Jéssica e Bibi agradeço toda a preocupação principalmente nesta fase final do meu percurso académico, foram sem dúvida um pilar.

Não poderia deixar de incluir um agradecimento especial à casa do povo por toda a animação 24/24h, todas tão diferentes, mas com o mesmo gosto pela ciência. Obrigada pelas noites de estudo sem dormir e pelas rugas que vocês me causam.

Às minhas colegas de mestrado Rebeca, Rafaela e Bia obrigada pela partilha de ciência e bons jantares de “quartas mágicas”.

À minha família um imenso e eterno obrigado por terem feito de tudo para que tivesse a melhor educação e que sempre me incentivaram a dar um passo em frente. Por serem a base de estabilidade, confiança, coragem para que pudesse alcançar os meus objetivos. Sem vocês o que consegui hoje, talvez não teria a mesma importância.

Resumo

As proteínas flavodiféricas (FDPs) têm sido identificadas como participantes nos mecanismos de resposta ao stress nitrosativo e oxidativo em microrganismos anaeróbios estritos e facultativos. Estas proteínas são compostas por um domínio contendo o centro catalítico diférrico e outro contendo um FMN. *Escherichia coli*, um patógeno humano, codifica uma FDP classe B (chamada flavorubredoxina (FIRd)), possuindo um centro rubredoxina extra. A FIRd de *E. coli* é a única FDP descrita como tendo NO como substrato principal. Desta forma, este trabalho teve como objetivo compreender o mecanismo catalítico da FDP de *E. coli* na redução de NO para gerar N₂O.

A FIRd foi caracterizada utilizando técnicas bioquímicas e espectroscópicas, nomeadamente espectroscopias de UV-visível e de Ressonância Paramagnética Eletrônica, o que permitiu obter dados utilizados na caracterização funcional da proteína. Os resultados dos ensaios de cinética rápida demonstraram que, em condições de turnover único, a FIRd tem uma maior velocidade de reação quando NO é utilizado como substrato quando comparado ao O₂. Além disso, os resultados apontam para um mecanismo de reação onde a redução de NO pela FDP de *E. coli* ocorre através da formação de um intermediário dinitrosil [(FeNO⁷)]₂. Os resultados corroboram os mecanismos de reação propostos por D. Kurtz para as enzimas redutoras de O₂ de *Thermotoga marítima* e *Treponema denticola*.

Estudos semelhantes foram também realizados para uma FDP específica para o oxigénio, a FDP de Class F de *Clostridioides difficile*.

Concluimos que a FDP de *E. coli* é mais reativa para NO que oxigénio, consequentemente permitindo que microrganismos sobrevivam no intestino humano e no meio ambiente, promovendo assim a sua patogenicidade. Além disso concluímos também que a ligação da proteína para o NO ocorre progressivamente com a formação de diferentes intermediários. Desta forma, com este trabalho esperamos contribuir para o conhecimento ao nível funcional para a FDP de *E. coli*, tornando possível compreender e elucidar alguns mecanismos já propostos para esta proteína.

Palavras-chave: Centro diférico, proteínas flavodiféricas, flavorubredoxina, mecanismos catalíticos, óxido nítrico.

Abstract

Flavodiiron proteins (FDPs) have been identified as participants in the response mechanisms to nitrosative and oxidative stresses in strict and facultative anaerobic microorganisms. These proteins are composed of a domain containing the differic catalytic center and another containing an FMN. *Escherichia coli*, a human pathogen, encodes for a class B FDP (named flavorubredoxin, FLRd), having an extra rubredoxin center. FLRd is the only FDP described to date as having NO as its main substrate. Thus, this work aimed to understand the catalytic mechanism of *E. coli* FDP reducing NO to generate N₂O.

FLRd was characterized using biochemical and spectroscopic techniques, namely UV-visible and Electron Paramagnetic Resonance spectroscopies, which allowed to obtain data used in the functional characterization of the protein. The fast kinetic experiments showed that, in single turnover conditions, FLRd has higher reaction rates when NO is used as substrate when compared with O₂. Furthermore, the results point to a reaction mechanism where the NO reduction by the *E. coli* FDP occurs through the formation of a dinitrosyl [(FeNO)⁷]₂ intermediate. The results support the reaction mechanisms proposed by D. Kurtz for the O₂ reducing enzymes from *Thermotoga marítima* and *Treponema denticola*.

Similar studies were performed for an oxygen selective FDP, the Class F FDP from *Clostridioides difficile*.

We conclude that *E. coli* FDP is more reactive for NO, consequently a more efficient elimination of the NO present allows microorganisms to survive in the human intestine and in the environment, thus promoting its pathogenicity. Furthermore, we also conclude that the binding of the protein to the NO occurs progressively with the formation of different intermediates. Thus, with this work we hope to contribute to the knowledge at the functional level of *E. coli* FDP, making it possible to understand and elucidate some mechanisms already proposed for this family of proteins.

Keywords: Diiron center, flavodiiron proteins, flavorubredoxin, catalytic mechanisms, nitric oxide.

Symbols and abbreviations

BSA- Bovine Serum Albumin

C. difficile- *Clostridioides difficile*

CAT- Catalase

E. coli- *Escherichia coli*

EPR- Electron Paramagnetic Resonance

FDP- Flavodiiron Protein

FDP_F- Class F FDP

FIRd- Flavorubredoxin

GOX- Glucose oxidase

IPTG- Isopropyl- β -D-thiogalactopyranoside

LB- Lysogeny Broth

M9- Minimum Salts Medium

NO- Nitric oxide

O₂- Oxygen

RNS- Reactive Nitrogen Species

ROS- Reactive Oxygen Species

SDS-PAGE- Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis

Tris- Tris(hydroxymethyl)aminomethane

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1 Introduction

1.1 Oxygen

Geological studies indicate that about 2.5 billion years ago, the first signs of the presence of significant amounts of oxygen on Earth atmosphere appeared, resulting in the Great Oxygenation Event (GOE). After a long time, the atmospheric oxygen levels stabilised at the present-day value of 21%. [1] While some organisms still live in anaerobic environments, throughout evolution other organisms developed mechanisms to survive exposure to oxygen, or even to use it as a fundamental final electron acceptor of cell metabolism. In fact, many present-day organisms use it as the final acceptor of cell respiration, being involved in the oxidative phosphorylation process for the synthesis of ATP. [2]

However, during oxygen reduction, potentially harmful reactive oxygen species (ROS) may be produced, particularly in the mitochondrial respiratory chain. ROS can be non-radical (e.g. hydrogen peroxide (H_2O_2)), radical ions (e.g. superoxide anion ($\text{O}_2^{\cdot-}$)), or neutral radicals (e.g. hydroxyl radicals ($\text{HO}^{\cdot}$)), depending on the number of electrons used in oxygen reduction. The first step in ROS production occurs with a one electron reduction of molecular oxygen, forming the superoxide radical anion ($\text{O}_2^{\cdot-}$), which is the precursor of most ROS. Then, $\text{O}_2^{\cdot-}$ may be reduced to produce hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water in a two-electrons reduction or partially reduced to hydroxyl radical ($\text{HO}^{\cdot}$) in a one-electron reduction [3], Figure 1.

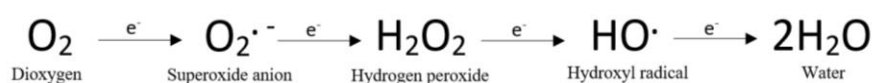
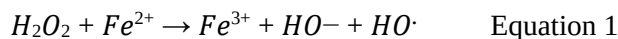


Figure 1: Reduction of oxygen and formation of ROS. Different ROS are formed by consecutive one-electron reduction: superoxide anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\text{HO}^{\cdot}$).

The production of ROS results from both enzymatic and nonenzymatic processes. For example, in the respiratory chain, phagocytosis processes, prostaglandin synthesis or in the cytochrome P₄₅₀ system, there are enzymes capable of producing ROS. As for non-enzymatic reactions, these generate free radicals as a by-product of the reaction of oxygen with organic compounds or when

cells are exposed to ionizing radiation. Furthermore, nonenzymatic free radical production can also occur during mitochondrial respiration.[4]

In addition, divalent metal ions, such as Fe^{2+} , may also be involved in Fenton chemistry, reacting with H_2O_2 to produce ROS, namely the hydroxyl radical, as shown in Equation 1 that, inside cells, may lead to different cellular damages.



Although aerobic organisms have a defence system to neutralize the effects of ROS, as these molecules are highly reactive, an imbalance between the formation of ROS and the cells' ability to eliminate them may give rise to a phenomenon known as oxidative stress. The increased production of ROS can interfere with many cellular structures such as membranes, lipids, proteins, lipoproteins and desoxyribonucleic acids (DNA), sometimes causing the appearance of pathological conditions, including cardiovascular diseases, diabetes, rheumatoid arthritis, cancer and neurodegenerative diseases.[4]

However, some studies demonstrate the importance of ROS in biological processes when kept in controlled concentrations, having an important role in homeostasis, cell signalling, regulation of metabolism and memory formation via DNA methylation.[3] In addition to having a beneficial role in the synthesis of some cellular structures, ROS are also used by the host's defence system to fight pathogens, having a central role in the innate immune system.[5]

1.2 Nitric oxide

NO is a free radical, structurally simple and has a low molecular mass [6]. NO is an uncharged lipophilic molecule containing a single unpaired electron [7] that makes it reactive with some molecules such as oxygen and reactive oxygen species (ROS), producing reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$), peroxynitrous acid (NHO_3) and nitrosothiols ($RSNO$). The reaction of NO and RNS may cause nitrosative damage on biomolecules and, consequently, affecting both cellular structures, namely proteins, and the function of the entire organisms.[6]

Biotically, nitric oxide is produced by two main routes: as an intermediate in dissimilatory denitrification in certain microbes and as the product of specialized enzymes for its synthesis, NO

synthases (NOS). For example in prokaryotes, NO is formed in the denitrification pathway by nitrite reductase (NIR) or as an intermediate in microbial anaerobic ammonium oxidation.[8]

NO synthesis by the NO synthases (NOS) requires 1.5 NADH/NADPH molecules and 2 molecules of oxygen (O_2) to convert its substrate, L-arginine, to L-citrulline and oxygen into NO [9], Figure 2. All NOS enzymes require the cofactor tetrahydrobiopterin (BH₄) to produce nitric oxide. In the absence of BH₄, NOS produce superoxide.[10]

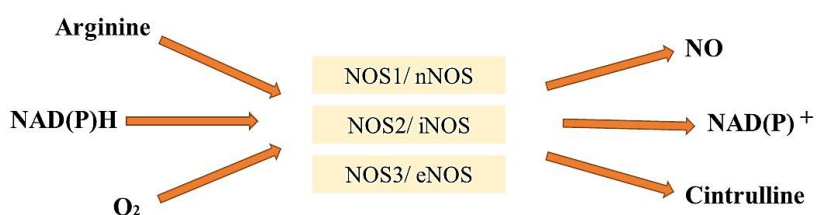


Figure 2: Production of NO and L-citrulline by NOS isoforms from L-arginine and oxygen.

NO is produced by three isoforms of NO synthases: NOS1, NOS2 and NOS3, having these NOS enzymes a critical role in a wide range of physiological contexts. Neuronal NOS (nNOS or NOS 1) is principally expressed in central and peripheral neurons. Endothelial NOS (eNOS, NOS 3) is mostly expressed in endothelial cells, keeping blood vessels dilated, regulates blood pressure, and has a variety of vasoprotective and anti-atherosclerotic effects. Inducible NOS (iNOS or NOS 2) can be expressed in many cell types, in mammals, for example, by cells of the innate immune system such as macrophages, in response to bacterial invasion [11], being involved in inflammatory diseases.[12] To initiate their activity, both nNOS and eNOS are calcium-dependent, contrary to iNOS which does not require a rise in calcium concentration to initiate its activity.[10]

At submicromolar concentrations, NO plays a key role as a signalling molecule, regulating physiological processes such as smooth muscle relaxation, neurotransmission, and blood pressure control.[8] However, at micromolar concentrations, NO is harmful, not just to bacteria cells but also to host cells, impairing a significant number of metabolic pathways.[13] This indicates that NO serves both protective and harmful functions in the body, depending on the cellular microenvironment and concentration.

Therefore, much research has been performed over the last thirty years that has revealed major biological roles for NO.[9]

As a result of the harmful role that NO may have to organisms, over millions of years of evolution these have developed many mechanisms that allow them to survive to the presence of NO and RNS. Organisms such as *Escherichia coli*, *Moorella thermoacetica*, *Paracoccus denitrificans*, *Pseudomonas nautica*, *Thermotoga maritima* and *Trichomonas vaginalis* have developed defence mechanisms that involve enzymes such as nitric oxide reductases (NOR), flavohaemoglobins and flavodiiron proteins (FDPs).[8]

1.3 Flavodiiron proteins

Flavodiiron proteins are a family of enzymes that exists in the three life domains: Archaea, Bacteria and Eukarya.[14] Flavodiiron proteins are characterized by the presence of a two-domain core: a N-terminal metallo- β -lactamase-like domain, containing the diiron catalytic center (Fe-Fe), where substrate reduction occurs, followed by a flavodoxin-like domain containing FMN in the C-terminal [15], as shown in Figure 3. These two domains represent the simplest form of this family of enzymes. However, in addition to these two domains, bioinformatics and biochemical studies have shown the existence of extra domains in the C-terminal part of the protein.[8]

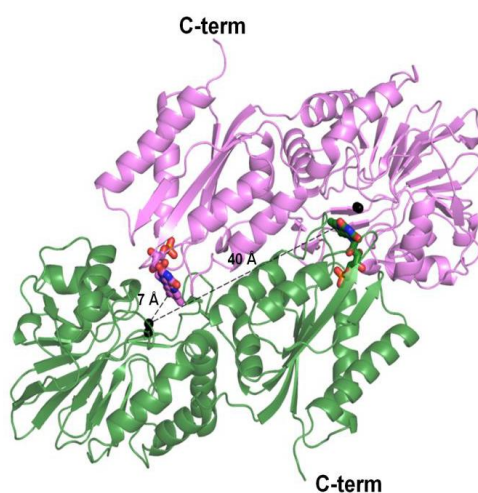
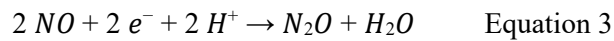
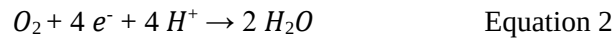


Figure 3: Cartoon representation of one FIRD monomer. In this representation the iron atoms are represented as black spheres and FMN as sticks with carbon in green or pink, nitrogen in blue and oxygen in red [15].

The first FDP was described in 1993 by Chen and co-workers [16] while studying the physiological role of oxygen in the sulfate reducing bacterium *Desulfovibrio gigas*. This protein, named rubredoxin-oxygen oxidoreductase (ROO), was described as an oxygen reductase, receiving electrons from rubredoxin while simultaneously oxidizing NADH without the release of reactive oxygen species (ROS).

Later, through bioinformatics and biochemical studies, Wasserfallen and co-workers [17] identified several type A flavoproteins (FrpA), homologues of the ROO from *Desulfovibrio gigas*. While most of the attention was focused on the role of FDPs in the oxygen detoxification, Gardner and co-workers [18] made an interesting observation: upon exposure to NO of anaerobically grown *E. coli* cells, they detected the induction of a ROO homologue, named flavorubredoxin. In the same year, Gomes and co-workers [19] demonstrated *in vitro* that indeed *E. coli* flavorubredoxin is a bona fide NO reductase, reducing NO to the innocuous N₂O. These works revealed the capacity of this family of proteins to perform the reduction of NO, besides oxygen. The NO or O₂ reduction activities of FDPs depend on still unknown structural factors: for example, the FDP from *Escherichia coli* (flavorubredoxin) preferentially uses NO as a substrate, with a turnover rate approximately 10 times higher than that for oxygen [19], while some FDPs from other organisms have activities for O₂ reduction up to 100 times higher than for NO reduction (e.g., FDPs from *Giardia intestinalis*[20], *Entamoeba histolytica*[21] and *Clostridioides difficile*[22]). Other FDPs have similar activities for both substrates (e.g, *Moorella thermoacetica*, *Desulfovibrio gigas* and *D. vulgaris*)[23].

Therefore, FDPs play a key role in oxygen and/or nitric oxide detoxification, catalysing the conversion of molecular oxygen to water and the conversion of nitric oxide to nitrous oxide and water [24], as shown in equations 2 and 3 respectively.



In addition to the FDPs having activities for both molecular oxygen and nitric oxide, in 2018 Folgosa and co-workers [22] showed that these proteins are also capable of reducing hydrogen peroxide to water using two-electrons, thus displaying hydrogen peroxide reductase activity.

1.4 Classes of FDPs

As mentioned above, this family of enzymes, flavodiiron proteins, are characterized by the presence of a flavodiiron core, that defines the family members. However, by bioinformatics and biochemical analysis more complex arrangements with other extra domains in the C-terminal part of the protein were identified. The FDP family can be divided into nine different classes from A to I, accordingly with the composition and distribution of their different structural domains [25], Figure 4.

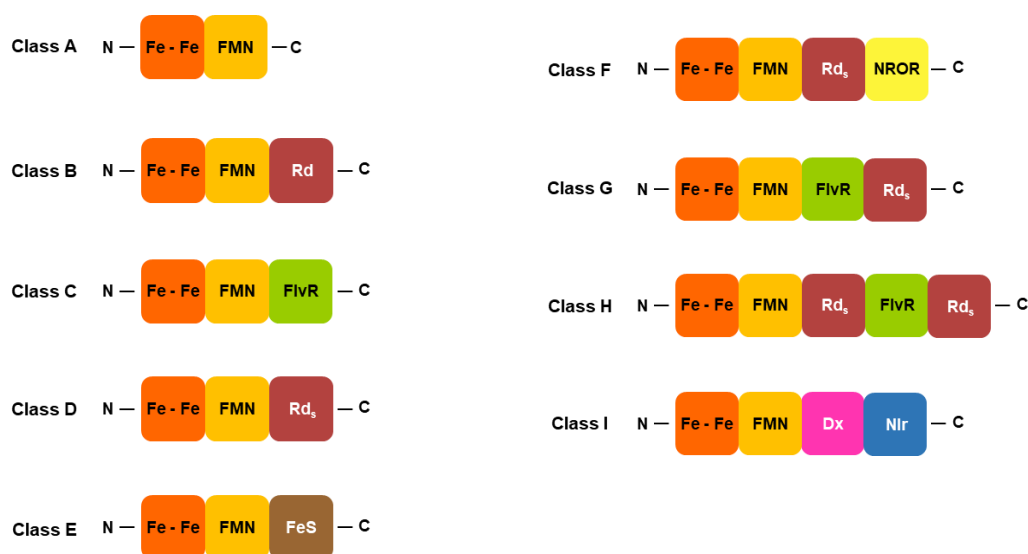


Figure 4: Organization of flavodiiron proteins in nine classes (class A-I). Fe-Fe (orange)- diiron center; FMN (golden)- flavodoxin like domain; Rd (red)- rubredoxin; FlvR (green)- NAD(P)H: flavin oxidoreductases; Rds (red)- short rubredoxin; FeS (brown)- iron sulphur cluster; NROR (yellow)- NADH:rubredoxin oxidoreductase; Dx (pink)- desulfiredoxin-like domain; Nir (blue) - neelaredoxin-like domain. Adapted from Martins, M. C. et al. [20].

The class A is the simplest one, containing only the flavodiiron core (the diiron center and the FMN containing domains). This class A FDP is mostly found in anaerobes and has been isolated from sulphate reducing bacteria (*D. gigas* and *D. vulgaris*)[16], few bacteria from the Firmicutes phylum (*Moorella thermoacetica* [26], *Clostridium acetobutylicum* [27] and *Clostridium difficile* [27]), methanogens (*Methanobrevibacter arboriphilus* [28] and *Methanothermobacter marburgensis* [29]), purple bacteria (*Rhodobacter capsulatus*)[17] and also from pathogenic unicellular protists (*Trichomonas vaginalis* [30], *Giardia intestinalis* [20] and *Entamoeba histolytica* [21]).[24] This is also the class of FDPs with the largest number of three-dimensional structures determined.

Class B FDPs, also named flavorubredoxin (FlRd), were first described in 1998 by Wasserfallen and co-workers [17] for the facultative anaerobe *E. coli*. Class B is composed by the presence of the common FDP core and a rubredoxin-like domain (canonical) linked to the C-terminal as an extra domain. This domain is involved in the electron transfer from the electron donor to the diiron center through a kinetically stable flavin semiquinone (FMN_{SQ}).[31] As mentioned above, this class B FDP from *E. coli* confers resistance to NO under anaerobic conditions, having an insignificant oxygen activity and therefore, it was classified as a NO reductase.[19]

Class C FDPs, (sometimes called Flvs), were also first described in 1998 by Wasserfallen and co-workers [17], for the FDP from *Synechocystis* sp. PCC6803, a freshwater cyanobacterium. This class is typical of photosynthetic organisms however, it was not found to date in any angiosperm genome nor in the genomes of red and brown algae. Class C FDPs contain, besides the flavodiiron core, an extra domain, a NADH(P)H: flavin oxidoreductase domain, that, unlike some classes of FDPs, has the capacity to receive electrons directly from NADH/NADPH. Prokaryotes, specifically the cyanobacterium *Synechocystis* sp. strain PCC 6803, provided the first evidence of Flv's activity in photosynthetic organisms, by demonstrating that there is an O₂ photo-reduction activity: these class C FDPs play an important role in the photoprotection of photosystems I and II in phototrophs, namely at low carbon dioxide levels or high and fluctuating light intensities.[32] [33]

Class F FDPs represent one of the most complex classes in this protein family, with a long predicted amino acid sequence (ca 850–880 residues). This class F FDP was first described in 2018 by Folgosa and co-workers [22], and is characterized by the presence of four domains, a flavodiiron core, followed by two extra domains: a short-type rubredoxin domain and a NADH:rubredoxin oxidoreductase (NROR) domain in the C-terminal part of the protein. In contrast to Class A and B FDPs, this protein does not require any other redox partner as it is able to receive electrons directly from NADH through its extra domains, NROR and rubredoxin.[22] [8]

In contrast to the four classes mentioned, no protein studies of the remaining five classes of FDPs were ever published to date. Therefore, their biochemical and spectroscopic properties are deduced from their amino acid sequences and structural predictions. Class D FDPs appear to be similar to Class B FDPs by presenting a rubredoxin domain in the C-terminal part. However, instead of the canonical rubredoxin domain observed in Class B, in Class D this domain is of the short type. Class E FDPs, found in *Clostridiales* genomes from the Firmicutes phylum, appear to possess an extra domain with a putative iron-sulfur center. The Class G FDPs, found mainly for the *Lactobacillales*, *Clostridiales* and *Erysipelotrichia*, are characterized by the presence of 2-3 extra domains: NADH(P)H: flavin oxidoreductase domain and 1 or 2 short-rubredoxin domains.

The Class H FDPs were described as having a composition similar to class G, presenting besides the flavodiiron core, a short-rubredoxin domain followed by NADH(P)H: flavin oxidoreductase domain, and by a second short-rubredoxin domain.[8]

More recently, a new class was proposed, Class I, from the strict anaerobe *Anaerofustis stercorihominis*. This class appears to have in its structure the flavodiiron core domain fused with a 2Fe superoxide reductase (SOR), having as extra domains: a desulfiredoxin (Dx) and a Neelaredoxin (Nlr) domains.[25]

1.5 Electron transfer process

The catalytic cycle of FDPs is a complex process due to the diversity of domains involved, namely the extra domains at the C-terminal, present in the nine classes A to I.[25] Therefore, it is particularly important to determine the electron transfer chains between the electron donors, NAD(P)H, and the terminal electron acceptor (O_2 and NO).[34]

Generally, the electron transfer pathway is initiated by the oxidation of NAD(P)H by an NAD(P)H oxidase which can be one of the domains of the FDP (in the case of Classes C, F and, most likely, G and H) [25] [22], or a redox partner (in the other classes) [16][17]. The electrons are then transferred from the NAD(P)H oxidase to the other domains, such as the rubredoxin (Class B)[17] or short-rubredoxin (Classes D, F, G and H)[25][22], Fe-S clusters (Class E)[25] or even to another redox partner, such as rubredoxins, as in Class A[16]. Subsequently, electrons are transferred to the flavin domain (FMN) and, at last, to the diiron site where the catalysis takes place. In addition to this, some Class A FDPs, instead of having NAD(P)H as electron donor, receive electrons from coenzyme F_{420} (in methanogens)[28] or ferredoxin[35], Figure 5.

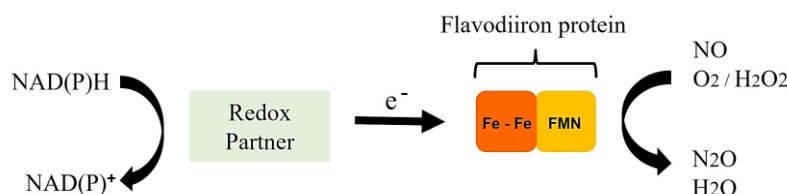


Figure 5: Schematic view of the general process of electron transfer proposed for the flavodiiron proteins. The catalytic mechanism of FDPs requires an electron donor (usually NAD(P)H) and reduces nitric oxide to nitrous oxide, and also oxygen and hydrogen peroxide to water.

1.6 Catalytic Mechanisms of NO reduction

FDPs perform the reduction of nitric oxide to nitrous oxide using NAD(P)H as a primary electron donor, in a process named nitric oxide reductase activity. To occur, this process requires the fully reduced active site and, if fully reduced (both the diiron site and FMN), FDPs can reduce up to four molecules of NO, generating two molecules of N₂O, resulting in the fully oxidized protein. [36]

To date, the class B FDP from *E. coli* is the only FDP identified with NO as its primary substrate, with a turnover rate approximately 10 times higher for NO than for O₂ [19]. Despite several studies, the reaction mechanism of these enzymes to reduce NO is still to be fully established. Besides, most of those studies were performed utilizing FDPs that are more reactive with O₂ than NO.

Nevertheless, four distinct mechanisms have been proposed for the reduction of NO to N₂O by FDP, which will be now briefly presented.

1.6.1 The “Super-reduction” mechanism

In 2005, Kurtz and co-workers proposed a reduction mechanism of NO by the FDPs from *M. thermoacetia* [37] and *D. vulgaris* [38], called the super-reduction mechanism. In this mechanism, the first NO molecule binds one of the iron atoms of the diiron species to form a diferrous mononitrosyl intermediate, [$\text{Fe}^{\text{II}}\text{NO}$]⁷. Then, the second NO molecule binds to the second iron atom, forming a diferrous dinitrosyl intermediate [FeNO]⁷₂ that is further “super-reduced” by FMNH₂ to form [$\text{Fe}(\text{HNO})$]⁸₂. In the last step, a water molecule and N₂O are released, and diferric state of the diiron active site is formed [39][40], Figure 6 a.

1.6.2 The “Hyponitrite” mechanism

In 2007, Siegbahn and co-workers [41] did computational research to propose an alternative reaction mechanism for FDPs that relied on the creation of a hyponitrite intermediate, also utilizing the FDP from *M. thermoacetica* as a model.

In the proposed hyponitrite mechanism, the computational results suggested that the first NO molecule binds only one iron atom (proximal iron, Fe_P or distal iron, Fe_D), preferentially to the Fe_D, forming a diferrous mononitrosyl ([$\text{Fe}^{\text{II}}\text{NO}$]⁷) intermediate. The second NO molecule

attacks the bound NO, forming a hyponitrite ($[\{Fe^{III} \{(u-N_2O_2)^{2-}\}Fe^{III}])$ intermediate. The hyponitrite intermediate undergoes protonation via His25, which changes its hydrogen bond from Glu83 to O₂. Then, the N-O bond is cleaved, forming the product N₂O, and a ferryl species in the proximal iron, Fe_P (IV)=O. The presence of two hydroxo bridges and the simultaneous decay of the ferryl iron, occurs subsequently, resulting in the formation of the diferric species. To reset the catalytic cycle, the diferric species is then reduced by FMNH₂ to regenerate the diferrous state of the diiron active site [11], Figure 6 b.

1.6.3 The “diferrous-dinitrosyl” mechanism

In 2010, Kurtz and co-workers [42] proposed a diferrous-dinitrosyl mechanism for the FDP from *M. thermoacetica*. In this mechanism is formed as first intermediate a diferrous-mononitrosyl, $Fe^{II}\{FeNO\}^7$, upon addition of less than 1 equivalent of NO per diferrous species. Contrary to the proposed hyponitrite mechanism, NO binding occurs preferentially to the proximal, Fe_P atom, interacting weakly with Fe_D. Then, binding of the second NO molecule generates the diferrous-dinitrosyl $[\{FeNO\}^7]_2$ intermediate, which subsequently releases N₂O and water. The diferric species is then reduced by FMNH₂ to regenerate the diferrous state [36] [11], Figure 6 c.

1.6.4 The semireduction mechanism (or the mixed-valence mechanism)

In 2018, Lehnert and co-workers [43] proposed a mechanism in which the diiron species is reduced stepwise by flavin (FMNH₂). This requires the flavin cofactor to act as a one-electron reductant instead of a two-electrons reductant towards the $[\{FeNO\}^7]_2$ intermediate, contrary to the mechanisms proposed and described above. In this semi-reduction mechanism, also named mixed-valence mechanism, two NO molecules bind the diiron center and form a $[\{FeNO\}^7]_2$ intermediate. Then, FMNH₂ gives one electron to reduce the $[\{FeNO\}^7]_2$ intermediate and generate a $[\{FeNO\}^7\{FeNO\}^8]$ species and FMNH(•), which subsequently leads to the production of one N₂O molecule and a mixed valent diiron (II,III) species. In the last step, FMNH(•) reduces the diiron (II,III) species to regenerate the diferric state of the diiron active site [11], Figure 6 d.

These proposed mechanisms are based on computational studies and experimental data that suggest the formation of a mononitrosyl pathway or the involvement of a dinitrosyl intermediate as a key intermediate, and these proposals remain to be experimentally proven. Furthermore, almost as controversial as the FDP reaction intermediates, is the role of the FMN in the protein, due to the proximity of this co-factor to the catalytic center.[44]

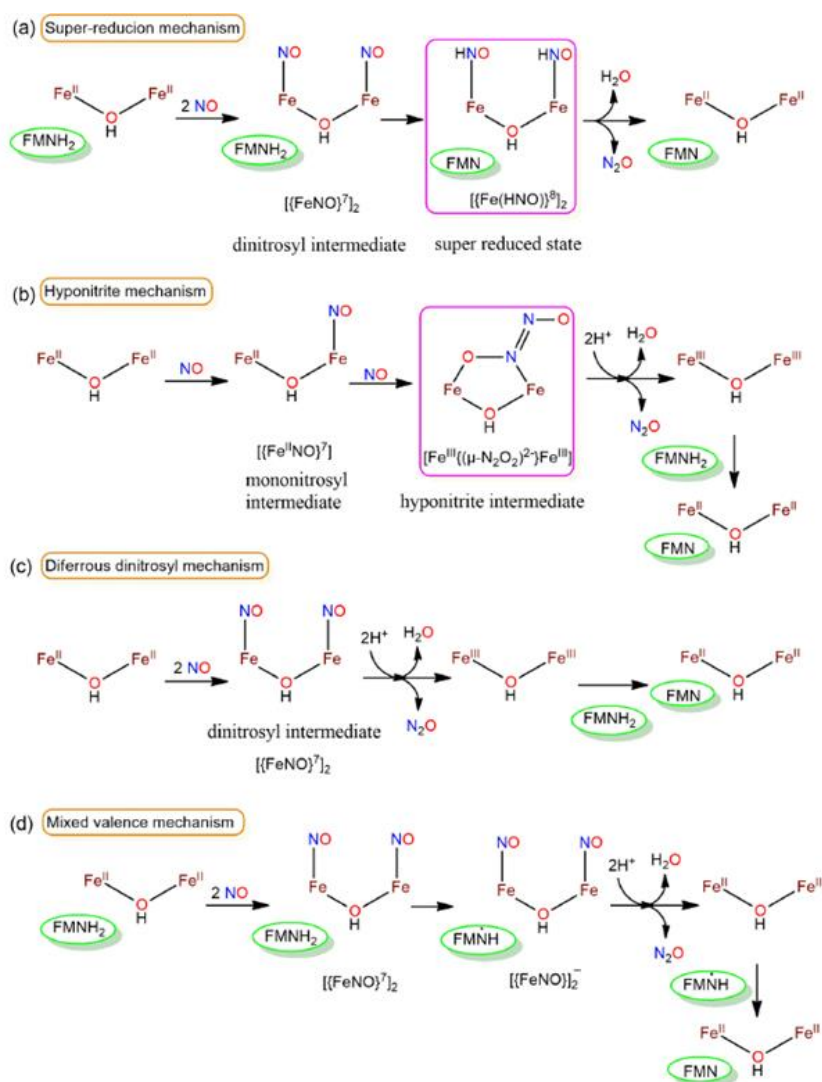


Figure 6: Proposed mechanisms for the two electrons reduction of NO to N₂O by FDPs. (a) super-reduction mechanism; (b) hyponitrite mechanism; (c) diferrous dinitrosyl mechanism and (d) mixed valence mechanism. Adapted from Majumdar Amit, et al. [40]

2 Aim

Flavorubredoxin, expressed by *Escherichia coli*, is the only FDP known to be a preferential NO reductase and one of the most investigated flavodiiron proteins. Nevertheless, the reaction mechanism of these enzymes regarding the reduction of NO is still undefined, as the FDPs investigated thus far have O₂ as the preferential substrate rather than NO.

Thus, this project aims to contribute to the knowledge of the reaction mechanism of FDPs, namely the one of *E. coli*, for the reduction of NO. To accomplish the main aim of this thesis, besides the spectroscopic and kinetic characterization of the *E. coli* FDP, also the one from *Clostridioides difficile*, that has oxygen as its preferential substrate, was studied.

3 Materials and methods

3.1 Cell transformation

The *E. coli norV* gene encoding for flavorubredoxin, as well as truncated genes encoding for flavodiiron protein domain (FDP core) and rubredoxin domain, the *E. coli norW* gene encoding for the flavorubredoxin reductase (FIRd Reductase) and of *cd1623* from *C. difficile* 630 encoding for the Class F FDP (FDP_F) were synthesized and cloned into the pET-24a expression plasmid by GenScript, USA. To obtain each protein, competent *E. coli* BL21(DE3) Gold cells were transformed with plasmid pET-24a containing the corresponding gene of interest as described in Appendix 8.1.

After transformation, cells were placed in LB-agar plates (composition in Appendix 8.2.1), supplemented with kanamycin 50 µg/mL, for FIRd, FDP core, FIRd Reductase and FDP_F or ampicillin 100 µg/mL for the rubredoxin domain, and incubated overnight at 37 °C, for selection of transformed cells.

3.2 Production of *E. coli* FDP and its mutants

Large scale cell growths were performed to obtain the proteins under study. Cultures used in protein expression started with a pre-culture of 300 mL in LB medium (composition in Appendix 8.2.2), containing kanamycin (50 µg/mL) and one colony of the *E. coli* cells transformed with the desired plasmid and left to grow overnight at 37 °C and 160 rpm. Afterwards, 8 L of minimal medium (M9) (composition in Appendixes 8.2.3 and 8.2.4) containing kanamycin (50 µg/mL) was prepared and inoculated with the addition of 3% of the pre-culture and supplemented with 100 µM of iron chloride (FeCl₂).

Cells were allowed to grow at 37°C and 160 rpm until an OD_{600nm} of 0.4. At this point, when the growth reached the exponential phase, expression of the desired protein was induced with 100 µM of isopropyl β-D-thiogalactopyranoside (IPTG), supplemented with an additional 100 µM of FeCl₂ and left to grow for 6 hours at room temperature. Cells were then harvested by centrifugation at 2300 g for 10 min at 4°C. Then, the pellet was resuspended in a 20 mM Tris-HCl pH 7.5 buffer and stored at -20 °C.

3.3 *E. coli* FDP purification

The cells suspension was incubated with DNase I (1 mg/mL) (Applichem) for 15 min and then disrupted in three cycles in a French-Press apparatus at 16000 psi (Thermo). After that, two centrifugation steps followed at 25000 g (Avanti J-25I, Beckman Coulter) for 30 min, 4 °C, and at 138000 g (Ultracentrifuge Optima LE-80K, Beckman Coulter) for 90 min at 4°C, to remove cell debris and the membrane fraction, respectively. The soluble fraction was dialysed overnight in a membrane dialysis (14kDa cut-off) in 20 mM Tris-HCl pH 7.5 buffer containing 18% glycerol, at 4°C. After the dialysis, the sample was centrifuged at 2300 g, 15 min at 4°C before loading into the chromatographic column.

Protein purification was performed by ion exchange chromatography in a Q-Sepharose Fast-Flow column (50 mL, GE Healthcare) previously equilibrated with 20 mM Tris-HCl pH 7.5 containing 18% glycerol connected to an ÄKTA Prime Plus system (GE Healthcare). The protein was eluted with a linear gradient from 0 to of 500 mM NaCl in 20 mM Tris-HCl pH 7.5 buffer containing 18% glycerol, in a total volume of 600 mL.

UV-Visible absorption spectroscopy on a UV-1700 Shimadzu was used to evaluate the resulting fractions. The purest fractions were pooled based on the ratio between the absorbances at 450 nm and 280 nm. After that, the selected fractions were evaluated by SDS-PAGE, as described in section 3.9.

Protein fractions collected were concentrated on Stirred Ultrafiltration Cells (Amicon) with a 30 kDa cut-off membrane. In this step, the buffer solution was changed to 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol and the sample was aliquoted and stored at -20 °C.

In the case of the FDP core, a second purification step was performed. The sample was loaded onto a Fractogel column (20 mL, Merck), previously equilibrated with 20 mM Tris-HCl pH 7.5 buffer containing 18% glycerol. The protein was eluted with a linear gradient from 0 to 500 mM NaCl in 20 mM Tris-HCl pH 7.5 buffer containing 18% glycerol, in a total volume of 400 mL.

The resulting fractions were again analysed by UV-Visible spectroscopy and the purest fractions were pooled based on the ratio between the absorbances at 450 nm and 280 nm. After that, the selected fractions were evaluated by SDS-PAGE, as described in Section 3.9.

Protein fractions collected were concentrated on Stirred Ultrafiltration Cells (Amicon) with a 30 kDa cut-off membrane. In this step, the buffer solution was changed to a 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol and the sample was aliquoted and stored at -20 °C.

3.4 Production of *E. coli* FDP rubredoxin

The *E. coli norV* gene truncated to encode for the rubredoxin domain was cloned into the pET-24a expression plasmid by GenScript, USA. Cultures used for protein expression started with a pre-culture of 200 mL of LB medium containing ampicillin (100 µg/mL) and one colony of the *E. coli* cells transformed with the desired plasmid and were left to grow overnight at 37 °C and 160 rpm. Afterwards, a 4 L of M9 culture medium containing ampicillin (100 µg/mL) was prepared and inoculated with the addition of 3% of the pre-culture and supplemented with 100 µM of FeCl₂.

Cells were allowed to grow at 37°C, 160 rpm until an OD_{600nm} of 0.8. At this point, expression of the desired protein was induced with 400 µM of IPTG, and the medium was supplemented with an additional 100 µM of iron chloride and left to grow for 4 hours at room temperature. Cells were then harvested by centrifugation at 2300 g for 10 min at 4 °C. Then, the pellet was resuspended in a 20 mM Tris-HCl pH 7.5 buffer and stored at -20 °C.

3.5 *E. coli* FDP rubredoxin purification

Initially, rubredoxin domain purification was done as described in section 3.3 for FlRd, but the resulting fractions were not satisfactorily purified, requiring another purification step. In the second purification step, the sample was loaded onto a Fractogel column (20 mL, Merck), previously equilibrated with 20 mM Tris-HCl pH 7.5, 18% glycerol. The protein was eluted with a linear gradient of 0 to 500 mM NaCl in 20 mM Tris-HCl pH 7.5 buffer containing 18% glycerol, in a total volume of 400 mL.

The resulting fractions were analysed by UV-Visible spectroscopy and the purest fractions were pooled based on the ratio between the absorbances at 490 nm and 280 nm. After that, the selected fractions were evaluated by SDS-PAGE, as described in Section 3.9.

Protein fractions collected were concentrated on Stirred Ultrafiltration Cells (Amicon) with a 3 kDa cut-off membrane. In this step, the buffer solution was changed to a 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol and the sample was aliquoted and stored at -20 °C.

3.6 *E. coli* FDP reductase production and purification

The expression and purification of the FIRD reductase was previously performed at the laboratory and used for the assays in this thesis.

3.7 *C. difficile* FDP production

Large scale cell growth was performed to obtain the protein under study. The transformation and the pre-inoculum processes were done as described in Sections 3.1 and 3.2. Then, 8 L of minimal medium (M9) culture containing kanamycin (50 µg/mL) was prepared and inoculated with the addition of 3% of the pre-culture and supplemented with 100 µM of FeCl₂.

Cells were allowed to grow at 37°C, 160 rpm until an OD_{600nm} of 0.4. At this point, expression of the desired protein was induced with 100 µM of IPTG and the medium supplemented with additional 100 µM of FeCl₂. After 6 h of growth, cells were harvested and lysed as described in Section 3.2.

3.8 *C. difficile* FDP purification

Initially, FDP_F purification was done as described in section 3.3 for FIRD, but the resulting fractions were not satisfactorily purified, requiring another purification step. In the second purification step, the sample was loaded onto a Resource column (6 mL, Merck), previously equilibrated with 20 mM Tris-HCl pH 7.5, 18% glycerol. The protein was eluted with a linear gradient of 0 to 500 mM NaCl in 20 mM Tris-HCl pH 7.5 containing 18% glycerol, in a total volume of 400 mL.

The resulting fractions were analysed by UV-Visible spectroscopy and the selected fractions were evaluated by SDS-PAGE, as described in Section 3.9.

Protein fractions collected were concentrated, buffer exchanged, and stored as described in Section 3.3.

3.9 SDS-PAGE

Protein production and purification efficacy were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN Electrophoresis System (BioRad). As molecular masses markers the NZYTech protein marker II was used (molecular masses from 11 to 96 kDa) as illustrated in Appendix 8.3.

Initially, samples were prepared by a 1:1 mixture of protein samples and loading buffer up to a total volume of 15 μ L and then heated at 95 °C for 10 min, while molecular markers were heated for 5 min. All SDS-PAGE analyses were performed in a 15% acrylamide-bis-acrylamide gel (composition in Appendixes 8.4.1 and 8.4.2) with 1.5 mm thickness. Subsequently, electrophoresis was carried out at 80 V for 15 min and then, at 180 V for 1 hour. Sample visualization was achieved by Coomassie Brilliant Blue protein staining (composition in Appendix 8.4.3) for approximately 30 min. Then, the gel was immersed in destaining solution (composition in Appendix 8.4.4) until the bands of the gel became visible.

3.10 Protein quantification

Pierce bicinchoninic acid (BCA) Protein Assay Kit is an assay that allows determining the amount of total protein present in the sample, through colorimetric detection. This method uses peptide bonds in the protein reducing Cu^{2+} to Cu^{1+} at a rate proportional to the amount of protein present in the sample. The binding of BCA with Cu^{1+} forms a complex that exhibits an absorbance maximum at 562 nm.[45]

For determination of the calibration curve 8 tubes with BSA (bovine serum albumin) were prepared with concentrations between 0 and 2 mg/mL, for a total volume of 50 μ L. To each tube was added 1 mL of a previously prepared BCA solution by mixing 50 parts of reagent A (Bicinchoninic acid and tartrate in an alkaline carbonate buffer) with one part of reagent B (4% copper sulphate pentahydrate). Protein samples' concentration were determined by preparing two different dilutions, in triplicate, for each sample. After 2h incubation with the BCA reagent, in the dark, the absorbance was measured by UV-Vis spectrophotometry (Shimadzu UV-1700 spectrophotometer) at 562 nm in disposable plastic cuvettes.

3.11 Iron quantification

The amount of iron present in the total protein sample was measured by a colorimetric method. In this assay, the reaction of Fe^{2+} with orto-phenanthroline leads to the formation of a reddish complex that exhibits an absorbance maximum at 510 nm. To determine the iron content in the sample, a calibration curve was built, preparing 8 tubes with increasing amounts of an iron standard solution (0.01 mg/mL). The molar absorptivity of the complex formed at 510 nm is $\epsilon_{510}=11,400 \text{ M}^{-1} \text{ cm}^{-1}$. [46]

The iron content of each sample was measured in duplicate. Either 100 μL or 150 μL of each sample were diluted to a final volume of 400 μL with milliQ water. 50 μL of 8 M HCl was added to each tube, followed by mixing in the vortex and incubation for 15 min at room temperature. Then, 50 μL of a solution of 80% (w/v) trichloroacetic acid (TCA) was added to precipitate the protein and the mixture was again homogenized in the vortex. All tubes were incubated for 30 more min at room temperature. After incubation, the samples were centrifuged for 5 min, at 13000 rpm. Subsequently, the pellet was discarded, and the supernatant was divided into two parts of 150 μL each. To each tube was added 400 μL of a 10% (w/v) hydroxylamine chloride solution and vortexed. Finally, 400 μL of a 0.3% (w/v) 1,10-phenanthroline solution was added and again the mixture was incubated at room temperature for 2 hours. The absorbance of the samples was then measured by UV-Vis spectrophotometry at 510 nm in disposable plastic cuvettes.

3.12 Flavin quantification

The determination of flavin present in the protein sample was performed by UV-Vis spectrophotometry at 450 nm. The absorbance spectrum was measured, and the flavin content was determined through the extinction coefficient, $\epsilon_{450}=12,500 \text{ M}^{-1} \text{ cm}^{-1}$. [47]

As for iron quantification, samples were prepared in duplicate. Either 100 μL or 150 μL of each sample were used and the final volume was adjusted to 400 μL with milliQ water. Then, 50 μL of 8 M HCl solution was added followed by 15 min of incubation at room temperature. Next, 50 μL of 80 % (w/v) TCA solution was added and the mixtures were incubated for 1 more hour at room temperature, followed by centrifugation at 13000 rpm for 5 min. Subsequently, the pellet was discarded, and the supernatant was divided into two parts of 150 μL each. To each tube was added 100 μL of a 75% (w/v) ammonium acetate to neutralize de pH of the solution and the final volume was adjusted to 500 μL with milliQ water. The UV-Visible spectrum of each sample was then measured at 450 nm.

3.13 Reduction and oxidation of protein with different substrates

The different reduction and re-oxidation profiles of the proteins under study were analysed by UV-Visible spectrophotometry (Shimadzu UV-1800 spectrophotometer). Spectra were collected anaerobically inside an anaerobic chamber (Coy Laboratory Products) with an O₂ level below 1 ppm. The protein samples and buffers were degassed prior to use and an O₂ scavenging system, composed by 10 mM of glucose, 375 nM of glucose oxidase (GOX) and 750 nM of catalase (CAT) was used, except for the assays that used O₂ as substrate for reoxidation. The concentrations of NADH and NADPH stock solutions were determined spectrophotometrically using the extinction coefficient $\epsilon_{340\text{nm}}=6220 \text{ M}^{-1} \text{ cm}^{-1}$. [48] The NO saturated solution (1.91 mM) was prepared by saturating, on ice, degassed buffer solution, 50 mM Tris-HCl pH 7.5 containing 18% (v/v) glycerol, with NO gas, previously bubbled in KOH and water.

In this assay, protein samples (30 μM for FIRd with 1 μM reductase; 30 μM for FDP core with 1 μM rubredoxin domain and 1 μM reductase; 25 μM for FDP_F) were gradually reduced by successive additions of 0.5 NADH equivalents until reaching the fully reduced state. In the case of FDP_F, a similar experiment was also performed with NADPH. After reduction, the different protein samples were incubated with successive additions of 0.5 NO or O₂ equivalents. The reoxidation of the samples was monitored by UV-Visible spectrophotometry.

3.14 Fast kinetics

Fast kinetics experiments were performed in a Stopped-Flow apparatus (KineticAsyst, Hi-Tech scientific). Stopped-Flow is a method to study fast reaction mechanisms in solution over timescales ranging from a few milliseconds to several minutes. In general, the solutions of two separated “driving syringes” are rapidly mixed together and then “stopped” in an observation cell called “mixing chamber”. This chamber is irradiated with light and the absorbance is measured by UV-Visible spectrophotometry as a function of time. After the acquisition, data are transferred to a computer to be analysed by global analysis algorithms using the software Kinetics Studio, Figure 7.

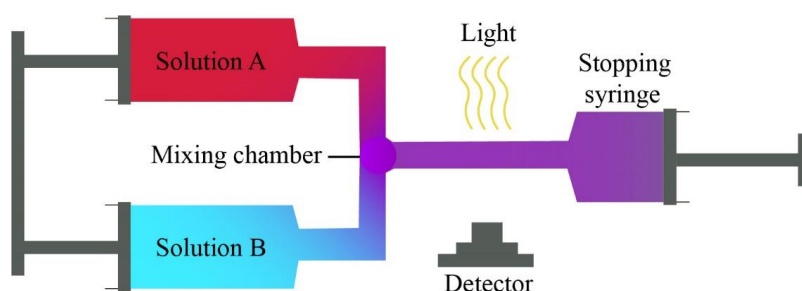


Figure 7: Schematic representation of the Stopped Flow apparatus. The direction of the flow is from left to right. The solutions are mixed in the mixing chamber and irradiated with light, which is then measured by a detector.

This method of analysing kinetic data can be used, for example, to calculate reaction rates, the complexity of the reaction mechanism, and may give information on reaction intermediates.

The protein reduction assays were prepared and executed under the conditions described in section 3.13. The fast kinetics assays were executed in a Stopped Flow apparatus inside an anaerobic chamber (mBraun) to keep the conditions as anaerobic as possible. The temperature of the entire apparatus was maintained at 15°C, and the buffer used was 50 mM Tris-HCl pH 7.5 containing 18% glycerol. As before, an O₂ scavenging system, composed by 10 mM of glucose, 375 nM of glucose oxidase (GOX) and 750 nM of catalase (CAT) was used, except for the assays that used O₂ as substrate for reoxidation.

Protein samples (60 µM for FIRd with 1 µM reductase; 60 µM for FDP core with 1 µM rubredoxin domain and 1 µM reductase; 60 µM for FDP_F) were previously reduced by stoichiometric addition of NADH inside a glove box. In addition, FDP_F was also reduced with stoichiometric amounts of NADPH.

In these assays, where the aim is to study reoxidation reactions of the proteins under study, FIRd, FDP core and FDP_F, with different substrates, the reoxidation was performed with air saturated buffer or NO saturated buffer as described before (section 3.13). The buffer solution was 50 mM Tris-HCl pH 7.5 containing 18% glycerol. These assays were performed by mixing the protein and the substrate solutions in a 1:1 ratio.

The parameters of fast kinetics were calculated from the fitting of the kinetic traces, in the Kinetics Studio program, the spectra obtained between 388 and 705 nm over time.

3.15 Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) spectroscopy is a useful biophysical technique for studying paramagnetic species, such as organic and inorganic radicals, as well as other paramagnetic species, that provides pertinent structural information about metalloproteins. EPR is based on the interaction of unpaired electron spins in a molecule with an external magnetic field. In an external magnetic field, unpaired electrons align with their magnetic momenta parallel (low energy) or antiparallel (high energy) to the magnetic field. When the resonance condition is achieved, energy is absorbed due to the transition between the low and high energy states. To obtain an EPR spectrum a variable magnetic field is applied at a given fixed microwave frequency (in the microwave region, for standard instruments).[49] [50]

To guarantee the absence of O₂ in the assays the protein samples and buffers were prepared under anaerobic conditions as described in section 3.13. Protein samples were prepared to final concentrations of 100 µM FIRd with 5 µM reductase; 150 µM FDP core with 5 µM reductase and 5 µM rubredoxin domain; and 83 µM FDP_F. Each protein sample was fully reduced by the stoichiometric addition of NADH. Fully reduced and partially reduced samples, with stoichiometric amounts of menadiol, were collected, frozen and stored in liquid nitrogen.

Then, re-oxidized protein samples were prepared by successive additions of NO saturated buffer. After each addition, the samples were incubated for 5 minutes, monitored by UV-visible, frozen and stored in liquid nitrogen.

X-band EPR spectra were obtained using a Bruker EMX spectrometer equipped with an Oxford Instruments ESR-900 continuous flow helium cryostat, and a high sensitivity perpendicular mode rectangular cavity. All spectra were recorded at 7 K, using a microwave frequency of 9.39 GHz, a modulation amplitude of 1.0 mT and a microwave power of 2 mW. EPR spectra were simulated using the program SpinCount.[51]

4 Results

4.1 Class B FDP from *Escherichia coli*, Flavorubredoxin

4.1.1 Biochemical characterization of *E. coli* FIRd

Flavorubredoxin, FIRd, was overexpressed in *E. coli* BL21(DE3) Gold and purified following the procedure previously described by Gomes and co-workers.[19] After the purification steps, the purity of the resulting fractions was assessed by UV-Visible spectroscopy and SDS-PAGE, Figure 8.

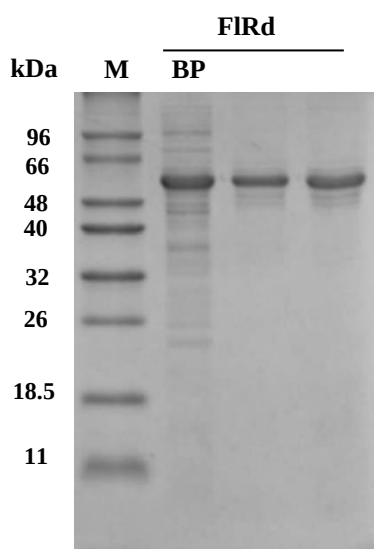


Figure 8: SDS-PAGE analysis of the purity of the FIRd fractions after the purification steps. The second lane corresponds to the *E. coli* soluble extract before the purification process (BP), the third and fourth lanes correspond to two different amounts of the final fraction of the pure protein, 0.5 μ g and 1 μ g samples. In the gel, the first lane (M) corresponds to the molecular mass markers (NZY protein marker II).

The SDS-PAGE presented in Figure 1 shows, as expected, in the lane correspondent to the *E. coli* extract, a variety of bands corresponding to all the proteins expressed. Considering the molecular mass predicted for FIRd monomers, 54 kDa, the intense band observed in SDS-PAGE gel indicates that the protein was successfully overexpressed. Although the final sample has a high degree of purity, a few bands of very low intensity due to possible contaminants are still observed.

The protein concentration of the final fraction was determined with the BCA method, and a value of 189 μ M FIRd was obtained. The iron content was measured by the orto-phenantroline method [27][52], yielding a 1.10 ± 0.01 Fe/monomer stoichiometry. This value is lower than the expected three atoms, two atoms for the diiron site and one atom in the rubredoxin domain. This result suggests that complete iron incorporation was not achieved despite the iron supplementation in the growth medium. Nevertheless, this iron content is within the range observed for other FDPs overexpressed in *E. coli*. [53], but it may be underestimated. A more precise determination using ICP would be needed.

The flavin content was measured by UV-Visible spectroscopy and determined as 0.38 ± 0.00 FMN/monomer. This value was also lower than expected as one flavin cofactor in the flavodoxin-line domain was not obtained. Since the FMN is non-covalently bound to the protein, the whole purification process from cell lysis until protein concentration may lead to the loss of the cofactor. Besides that, factors such as temperature fluctuation and exposure to light may cause degradation and flavin photoreduction, respectively. However, other flavodiiron proteins described in the literature have similar levels of FMN incorporation when overproduced in *E. coli*. [53]

The UV-Visible spectrum of FIRd in the as-isolated state shows the characteristic features of the domains containing FMN and the rubredoxin center: absorbance maxima at 377 and 470 nm, typical of oxidized flavin, and a broader band at 570 nm, resulting from the rubredoxin center contribution, as shown in Figure 9.

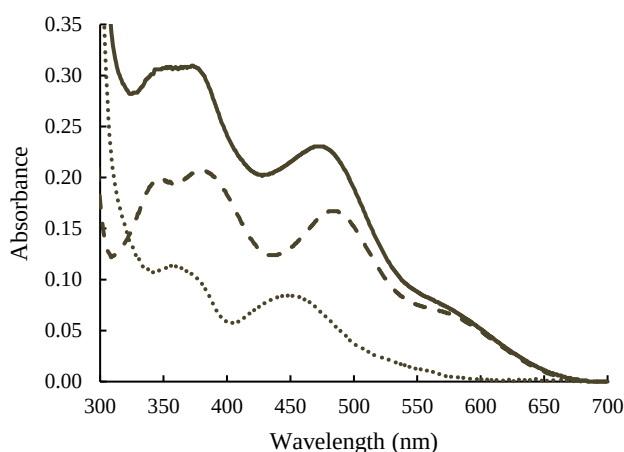


Figure 9: UV-Visible spectrum of FIRd. The black solid line represents the FIRd spectrum in the as isolated (oxidized) state, while the black dashed line and black dotted line represent the rubredoxin and FMN contributions, respectively. The FMN spectrum results from the subtraction of the rubredoxin spectrum contribution from the FIRd spectrum. Concentrations: [FIRd]= 37 μ M; [Rd-domain] = 30 μ M, in 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

4.1.2 Optimization of the *E. coli* FIRd-NO binding conditions

The reduction of FIRd with sequential sub-stoichiometric amounts of NADH as the electron donor was monitored by UV-Visible and allowed us to deconvolute the differential reduction of the FMN and rubredoxin center. Also, these experiments allowed us to establish the conditions necessary to prepare the EPR samples (see next Section).

By UV-Visible spectroscopy it was determined that the addition of 1.25 equivalents (eq) of NADH (in the presence of 1 μ M of FIRd reductase) leads to the complete bleach of the FMN absorption, observed by the decrease in the intensity of the bands at 380 and 450 nm, while further addition of NADH up to 2 eq. leads to a total disappearance of components of the spectrum, Figure 10, indicating that the rubredoxin center was also totally reduced.

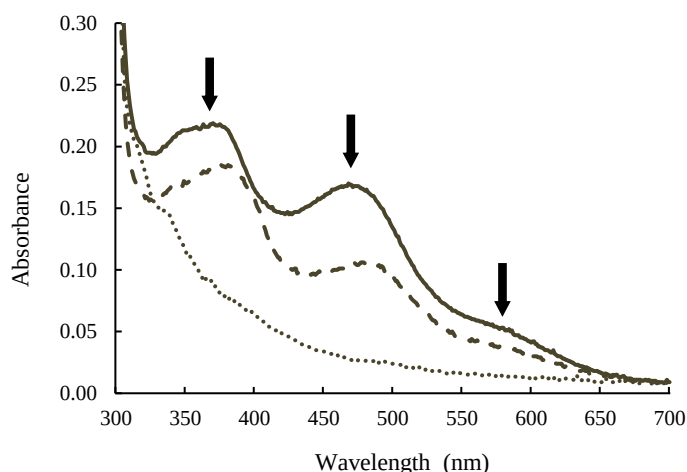


Figure 10: FIRd reduction by NADH. The black solid line represents the as-purified FIRd spectrum; the black dashed line represents the spectrum of the protein incubated with 1.25 eq. of NADH and the black dotted line represents the spectrum of the protein incubated with 2 eq. of NADH. Arrows point to the absorbance changes at different wavelengths. Concentrations: [FIRd]= 30 μ M; [Reductase]= 1 μ M, in 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

To assess the oxidation process with the substrate, NO was added and the changes in the UV-Visible spectrum were monitored. The protein was first fully reduced by the addition of 2 eq. of NADH, similarly as before, and as shown in Figure 11 A (black line). After reduction, the re-oxidation of the protein was performed by adding increasing amounts of NO, Figure 11. The addition of 1.5 eq. of NO leads to the formation of the semiquinone species of FMN, observed by the increase in the absorption at 366 nm and at 490 nm. A small increase in the absorbance is also observed at 570 nm, corresponding to the partial oxidation of the rubredoxin center. The addition

of 2.5 eq. of NO leads to fully oxidized FlRd with a spectrum similar to that of the as purified as shown in Figure 11 A.

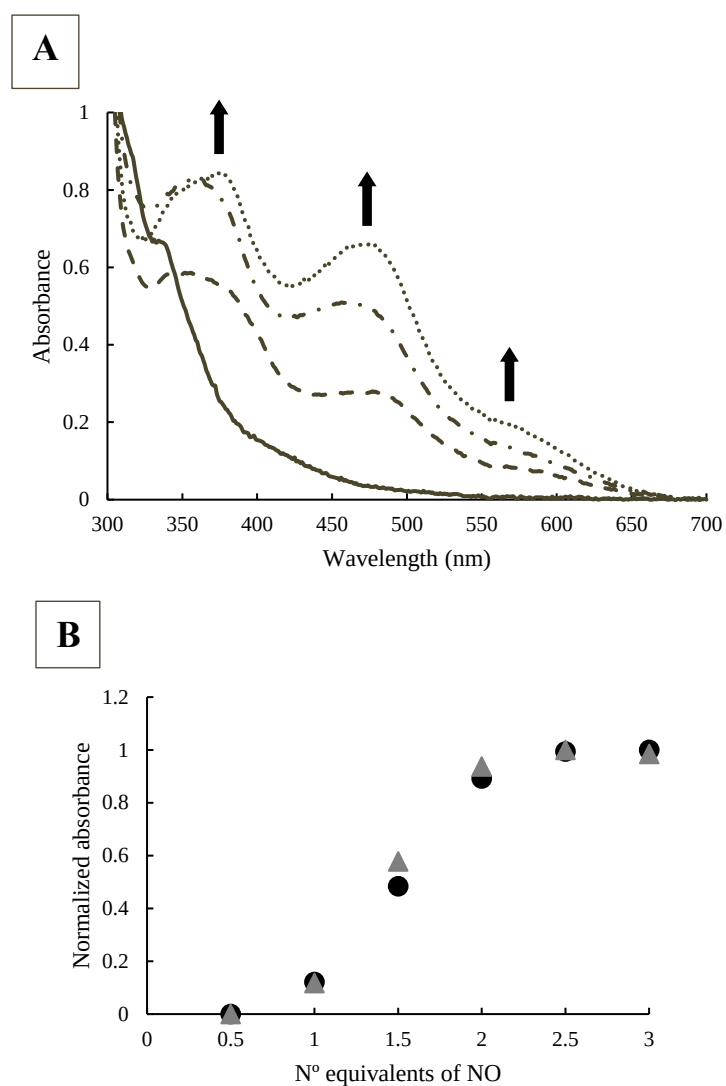


Figure 11: FlRd re-oxidation by NO. Panel A- The black dotted line represents FlRd in the as-isolated state; the black solid line represents the spectrum after the addition of 2 eq. of NADH; the black dashed line represents the spectrum after the addition of 1.5 eq. of NO and the black dashed and dotted line represents the spectrum after the addition of 3 eq. of NO. Arrows point to the absorbance changes at different wavelengths. Panel B- The black dots represent the normalized absorbance at 460 nm and the grey triangles represent the normalized absorbance at 570 nm after each addition of NO. Concentrations: [FlRd]= 100 μ M; [Reductase]= 5 μ M, in 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

4.1.3 *E. coli* FIRd-NO binding monitored by EPR

EPR is a useful technique to study metallic centres of metalloproteins, namely FDPs such as the FIRd of *E. coli*. In this case, the aim was to monitor the binding of NO to the catalytic diiron center of the *E. coli* FIRd.

The EPR spectrum of the fully reduced FIRd revealed, as expected, no EPR signal for the diiron site ($\text{Fe}^{\text{II}}\text{-Fe}^{\text{II}}$), as the iron ions are antiferromagnetically coupled, resulting in a total spin system $S=0$, or ferromagnetically coupled, with $S=4$. In addition, this also demonstrates that the rubredoxin center is in the reduced state, with $S=2$. As was observed in the UV-Vis spectroscopy experiments, the incubation with 1 eq. NO leads to the partial oxidation of the rubredoxin center, giving rise to a rhombic signal with g -values of 4.26 and 9.20 (minor), which correspond to the middle $|\pm 3/2\rangle$ and lower $|\pm 1/2\rangle$ Kramer doublets of the $S=5/2$ ferric state. Besides the contribution of the rubredoxin center, a rhombic signal is observed with g -values of 1.96, 1.81 and 1.78, which is typical of the diiron center in the mixed valence ($\text{Fe}^{\text{III}}\text{-Fe}^{\text{II}}$) state, with a total spin of $S=1/2$. As observed in Figure 12, the intensity of this signal is maximal when the protein is incubated with 3 eq. of NO and disappears after incubation with 4 eq. of NO, demonstrating that the fully oxidized ($\text{Fe}^{\text{III}}\text{-Fe}^{\text{III}}$) form of the diiron site was obtained. Furthermore, another signal at $g = 2.04$ is observed upon incubation with 3 eq. NO, as revealed in Figure 12 B. This signal is typical of iron-dinitrosyl complexes. The intense signal at $g=2.0$ is due to the semiquinone form of the FMN.

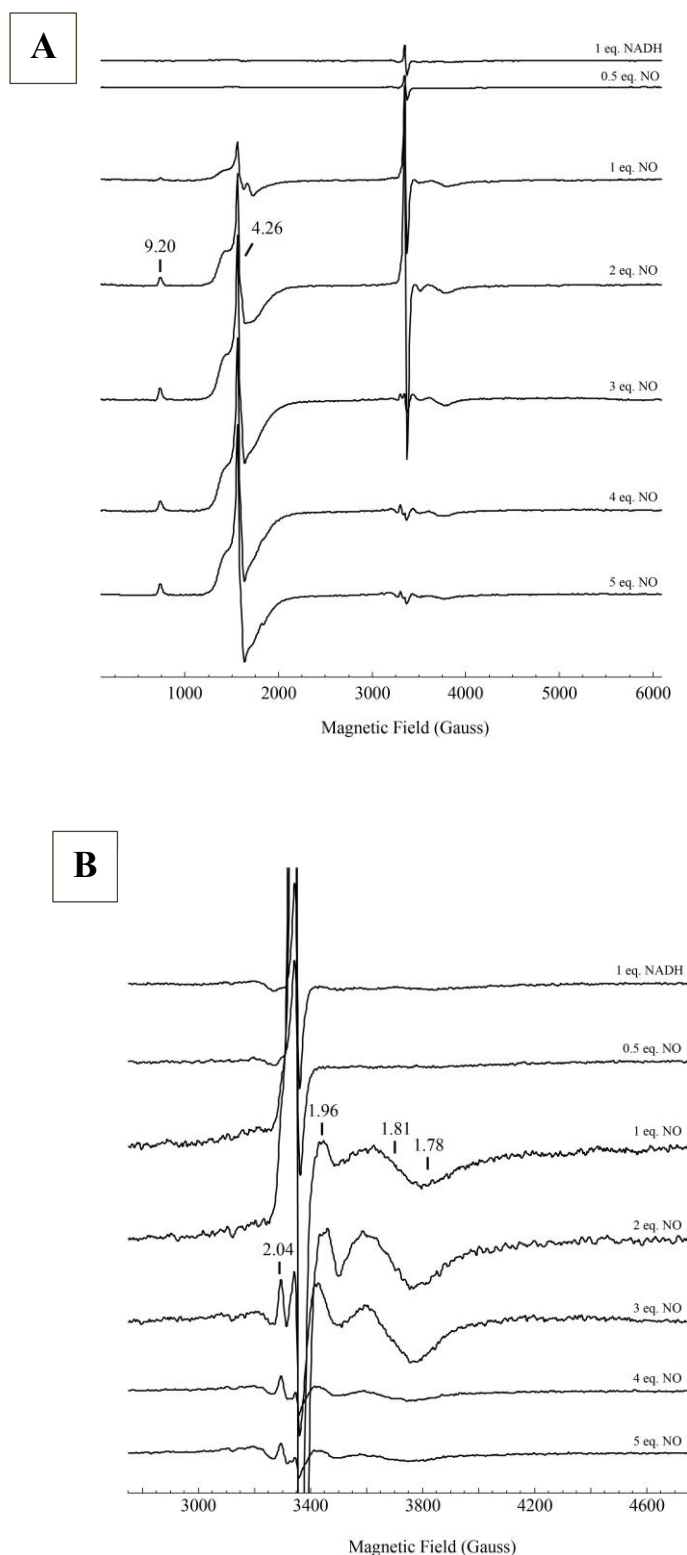


Figure 12: EPR spectra of F1Rd incubated with NO. Panel A- EPR spectra of the F1Rd after reduction with 1 eq. of NADH and addition of different amounts of NO (0.5 eq. until 5 eq. of NO). Panel B- EPR spectra of the same samples as described above collected in a spectral window centered in the diiron center signal. Arrows indicate the g values assigned to each signal. Concentrations: [F1Rd]= 100 μ M, [Reductase]= 5 μ M, in 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol. The spectra were collected at T= 7 K; microwave power 2 mW and microwave frequency= 9.39 GHz.

4.1.4 Study of the reaction of *E. coli* FIRd with NO by stopped-flow spectroscopy

Stopped-flow is a technique widely used to study reactions that develop in a very short time scale (down to the millisecond time range). The reaction of the *E. coli* FIRd with NO was investigated by stopped-flow spectroscopy under anaerobic conditions at 15°C in single turnover conditions. The protein sample was previously reduced with a stoichiometric amount of NADH to avoid multiple turnovers, similarly as before and as shown in Figure 11 A (black line). Then, reduced FIRd (60 μ M, 30 μ M after mixing) was mixed in a 1:1 ratio with NO saturated buffer (1.91 mM, 0.955 mM after mixing) and the reaction was monitored for different times scales (0.45s and 45s), Figure 13. The first spectrum, recorded at 0.75 ms, already shows a broad band at 450 nm which indicates a small amount of oxidized FMN. The spectra acquired at 0.45 s and 45 s, reveal the formation of oxidized FMN, characterized by an absorbance increase with a broad absorption band at 450 nm.

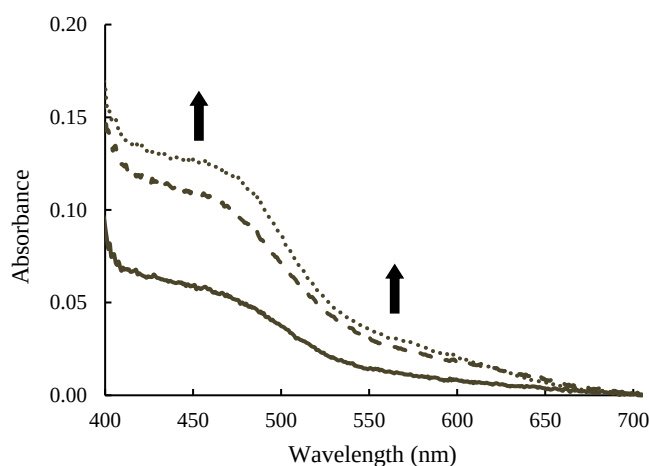


Figure 13: Reaction of FIRd (reduced by NADH) with NO followed by stopped-flow spectroscopy. Panel A- The black solid line represents the first spectrum collected at 0.75 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used in the analysis. Concentrations after mixing [FIRd]= 30 μ M; [Reductase]= 0.5 μ M; [NO]= 955 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

In order to estimate the reaction rates, the absorbances at 450 and 540 nm were followed over time, in two different time scales, 0.45 s and 45 s, as shown in Figure 14. Upon reaction with NO, an increase of absorbance at 450 nm was observed during 45 s, Figure 14 B, with a faster rate observed in the first 0.45 s of the assay, as shown in Figure 14 A and B. The reaction was also followed at 540 nm, Figure 14, to monitor the oxidation of the rubredoxin center. The reaction with NO led to an increase in the absorbance at 540 nm in the first 0.45 s, Figure 14 A.

As the NO or O₂ concentration used in the assay was much larger than the enzyme one, a pseudo-first order kinetics was assumed as illustrated below:

$$v = k [\textit{Substrate}][\textit{FDP}] \quad \text{Eq. 4}$$

$$[\textit{Substrate}] \gg [\textit{FDP}]$$

$$k' = k[\textit{Substrate}] \quad \text{Eq. 5}$$

$$v = k'[\textit{FDP}] \quad \text{Eq. 6}$$

As two different exponentials are observed, one very fast and the other slower, the kinetic traces were fitted with a sum of two exponentials, as shown in equation 7, considering the pseudo-first order kinetics described in equations 4, 5 and 6.

$$[\textit{FDP}]_{ox} = [\textit{FDP}]_1 e^{-k'_1 t} + [\textit{FDP}]_2 e^{-k'_2 t} \quad \text{Eq. 7}$$

The rate constants obtained were calculated based on triplicates and are summarized below in Table 1.

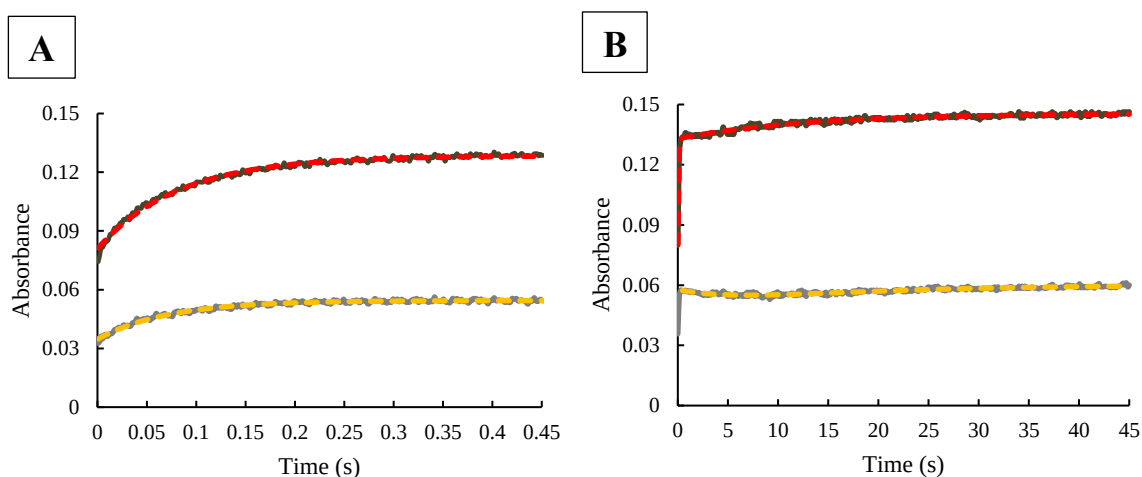


Figure 14: Typical traces and fits at 450 nm and 540 nm of F1Rd (reduced by NADH) reacted with NO followed by stopped-flow spectroscopy. Panel A- The black solid line represents the absorbance variation at 450 nm in the 0.45 s window assay. The fit to the curve is represented by the red dashed line. The grey solid line represents the variation in the absorbance at 540 nm in the 0.45 s assay. The fit to the curve is represented by the orange dashed line. Panel B- The black solid line represents the absorbance variation at 450 nm in the 45 s window assay. The fit to the curve is represented by the red dashed line. The grey solid line represents the absorbance variation at 540 nm in the 45 s assay. The fit to the curve is represented by the orange dashed line. Concentrations after mixing [F1Rd]= 30 μ M; [Reductase]= 0.5 μ M; [NO]= 955 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 1: Calculated reaction rates of the F1Rd (reduced by NADH) reacted with NO and followed at 450 nm and 540 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm and 540 nm over time *Concentration after mixing.

Wavelength (nm)	[NO] (μ M)*	k'_1 (s^{-1})	k'_2 (s^{-1})
450	955	12.2 ± 0.1	0.08 ± 0.02
540	955	13.7 ± 0.6	0.05 ± 0.02

The fact that the rate constants calculated for both wavelengths are similar within the error margin, indicate that we are observing the same processes, dominated by the oxidation of the rubredoxin. Similar experiments were performed using a 10x dilution of the NO saturated buffer as shown in Figure 15. The spectrum at 45 s reveals the formation of the oxidized rubredoxin center, characterized by absorption at 480 nm and >500 nm. By subtracting the spectrum at 45 s of the NO saturated buffer (1.91 mM, 0.955 mM after mixing) experiment to the 45 s spectrum of the 10x dilution of NO saturated buffer (0.191 mM, 0.0955 mM after mixing), it was possible to estimate the optical contribution of the 7 μ M rubredoxin domain in the fully oxidized protein. This result also shows that the reaction, in single turnover conditions, with the NO saturated buffer may lead to the partial degradation of the protein, namely the rubredoxin center.

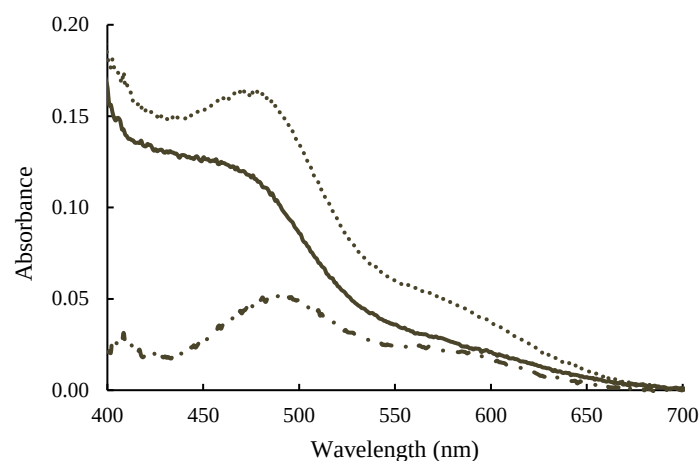


Figure 15: Reaction of FIRd (reduced by NADH) with different concentrations of NO followed by stopped-flow spectroscopy. The black solid line represents the spectrum of FIRd incubated with 955 μM NO at 45 s. The black dotted line represents the spectrum of FIRd incubated with 95.5 μM NO at 45 s. The black dashed and dotted line represents the spectrum of the rubredoxin domain obtained by subtraction of the two spectra. Concentrations after mixing [FIRd]= 30 μM ; [Reductase]= 0.5 μM ; [NO]= 955 μM and 95.5 μM ; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Upon reaction with 1.91 mM NO, 0.0955 mM after mixing, the spectral evolution was monitored. The first spectrum, acquired at 0.75 ms, already shows that the reaction with NO led to the appearance of the absorbance band with maximum at 450 nm, indicative of a partially oxidized FMN. The absorbance of this band increases over time appearing fully oxidized at 45 s, also observed by the absorption band at 570 nm, Figure 16.

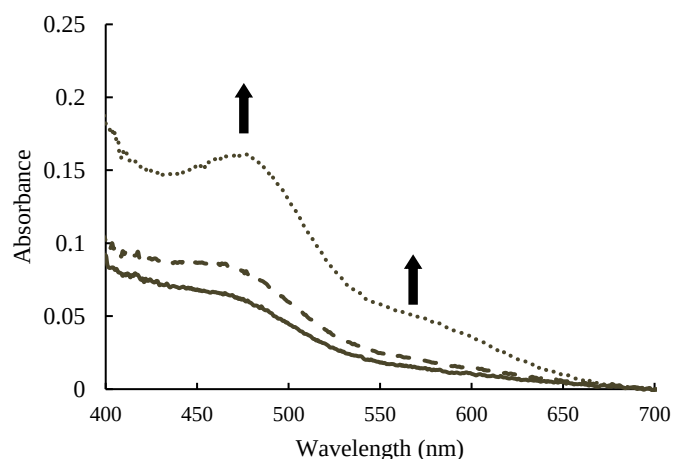


Figure 16: Reaction of FIRd (reduced by NADH) with NO followed by stopped-flow spectroscopy. Panel A- The black solid line represents the first spectrum collected at 0.75 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for the analysis. Concentrations after mixing [FIRd]= 30 μ M; [Reductase]= 0.5 μ M; [NO]= 95.5 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

The 10 x NO diluted solution allowed us to apply a pseudo-first order kinetic approach. Thus, the absorbance was monitored over time at 450 nm, as shown in Figure 17. Addition of NO led to an increase in absorbance at 450 nm, with a faster process in the first 7 s of the reaction, Figure 17 A and B. Therefore, the reaction rates were calculated by fitting of one exponential function, and respective rate constants are summarized below in Table 2.

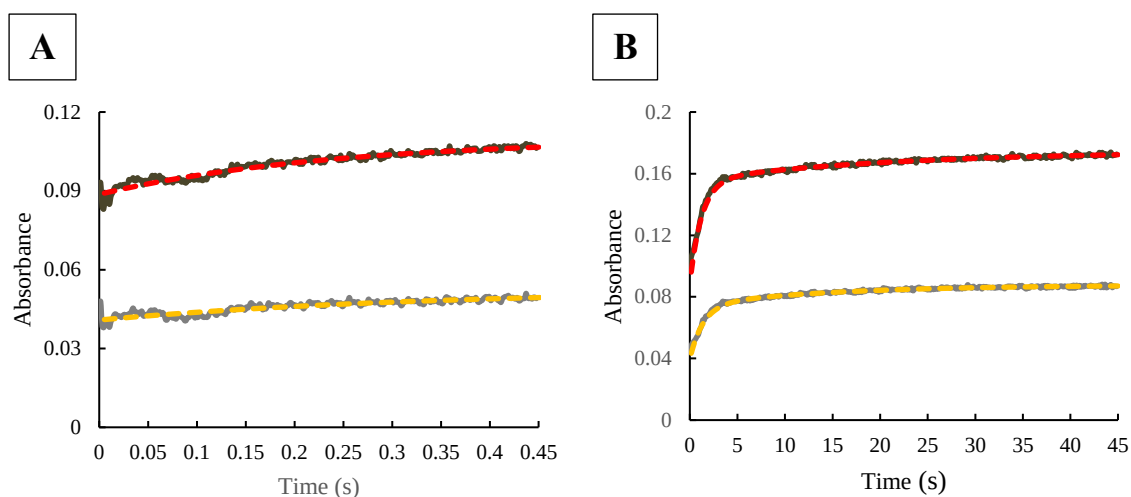


Figure 17: Typical traces and fits at 450 nm and 540 nm of FIRd (reduced by NADH) reacted with NO followed by stopped-flow spectroscopy. Panel A- The black solid line represents the absorbance variation at 450 nm in the 0.45 s window. The fit to the curve is represented by the red dashed line. The grey solid line represents the absorbance variation at 540 nm at 0.45 s assay. The fit to the curve is represented by the orange dashed line. Panel B- The black solid line represents the absorbance variation at 450 nm in the 45 s assay window. The fit to the curve is represented by the red dashed line. The grey solid line represents the absorbance variation at 540 nm in the 45 s assay. The fit to the curve is represented by the orange dashed line. Concentrations after mixing [FIRd]= 30 μ M; [Reductase]= 0.5 μ M; [NO]= 95.5 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 2: Calculated reaction rates of the FIRd (reduced by NADH) reacted with NO and followed at 450 nm and 540 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm and 540 nm over time *Concentration after mixing.

Wavelength (nm)	[NO] (μ M)*	k'_1 (s^{-1})	k'_2 (s^{-1})
450	95.5	-----	4.5 ± 0.5
540	95.5	-----	3.5 ± 0.9

As before, the fact that the calculated rate constant, k'_2 , determined for both wavelengths are similar within the error margin, indicating that the final process is dominated by the oxidation of rubredoxin. On the other hand, the FMN is already partially oxidized in the first spectrum at 0.75 ms, indicating that this process is defined by very high rate constant (k'_1) and, therefore, occurs in the dead-time of the experiment, and impossible to calculate.

4.1.5 Study of the reaction of *E. coli* FIRd with O₂ by stopped-flow spectroscopy

Similar experiments were performed using O₂ as substrate. As before, FIRd was previously reduced in anaerobic conditions with a stoichiometric amount of NADH, as shown in Figure 11 A (black line). Then, reduced FIRd was mixed with air-equilibrated buffer (~260 μ M O₂) in a 1:1 ratio. The first spectrum acquired at 2.26 ms does not exhibit any different spectral feature from the reduced FIRd. At 0.45 s, a broader band appears at 450 nm, indicative of a partially oxidized flavin co-factor, and at 45 s the fully oxidized FIRd is observed, with the appearance of the absorption bands at 474 nm and 570 nm, Figure 18.

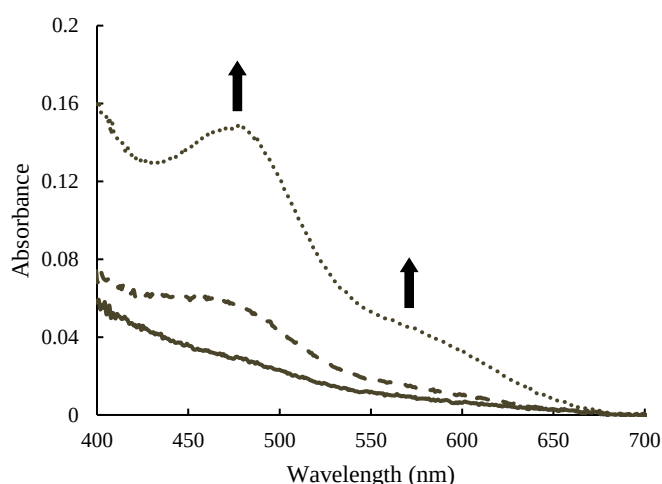


Figure 18: Reaction of FIRd (reduced by NADH) with O₂ followed by stopped-flow spectroscopy. The black solid line represents the first spectrum collected at 2.26 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for analysis. Concentrations after mixing [FIRd]= 30 μ M; [Reductase]= 0.5 μ M; [O₂] = 130 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Like in the reaction with NO, the experimental conditions used allow to apply a pseudo-first order kinetics approach. The analysis was performed by monitoring the absorbance at 450 and 540 nm over time, as shown in Figure 19. The reaction of the reduced FIRd with the air-equilibrated buffer led to an exponential increase in the absorbance at 450 nm during 45 s until the fully oxidized FIRd is obtained, Figure 19 B. At both wavelengths two steps were observed, one faster, only observed in the experiments recorded in the shorter time scale and a second one, slower, observed in the experiments recorded in a longer timescale, Figure 19 C. The fastest process is observed only in the 450 nm trace, corresponding to a $k' = 121.4 \text{ s}^{-1}$, indicating that the FMN is the first

cofactor to become oxidized after reaction with the substrate, most likely reducing the diiron catalytic center to allow the progress of the reaction. On the other hand, the slowest processes are observed at both wavelengths with $k' = 3.2 \text{ s}^{-1}$ at 450 nm and $k' = 6.7 \text{ s}^{-1}$ at 540 nm with similar rate constants. As was observed for the reaction with NO, this also indicates that the rubredoxin is the last center to be oxidized, contributing to both wavelengths in a similar fashion. The data was fitted with the sum of two exponential functions for the absorbance at 450 nm, as described in equation 7, and it was fitted with one exponential function for the absorbance at 540 nm, as summarized below in Table 3.

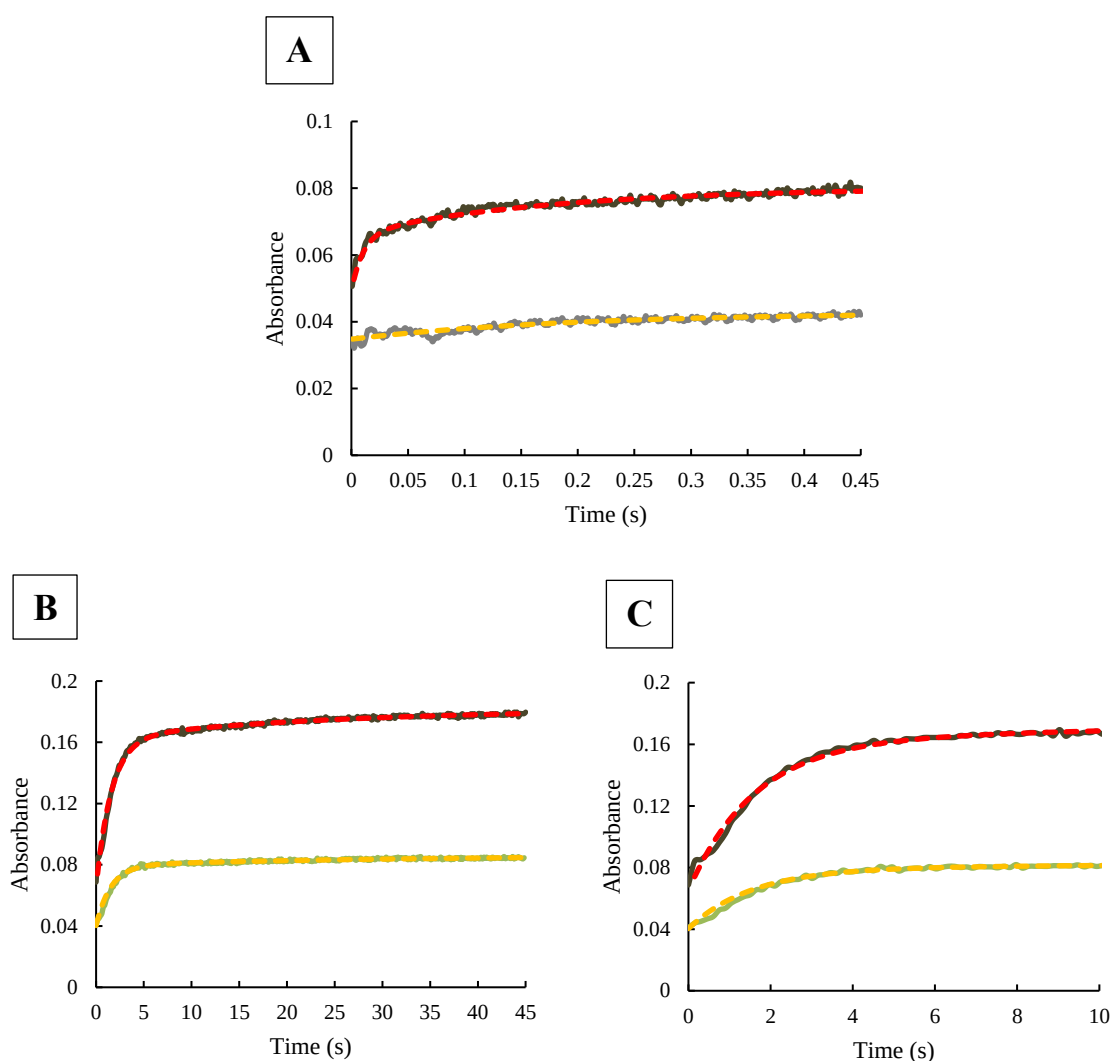


Figure 19: Typical traces and fits at 450 and 540 nm of FIRd (reduced by NADH) reacted with O₂ followed by stopped-flow spectroscopy. Panel A- The black solid line represents the variation absorbance at 450 nm in the 0.45 s window. The fit to the curve is represented by the red dashed line. The grey solid line represents the absorbance variation at 540 nm in the 0.45 s window. The fit to the curve is represented by the orange dashed line. Panel B- The black solid line represents the absorbance variation at 450 nm in the 45 s assay. The fit to the curve is represented with red dashed line. The grey solid line represents the variation absorbance at 540 nm in the 45 s window. The fit to the curve is represented by the orange dashed line. Panel C- It is an amplification of panel B until 10 s. Concentrations after mixing [FIRd]= 30 μM ; [Reductase] = 0.5 μM and [O₂] = 130 μM ; T = 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 3: Calculated reaction rates of the FlRd (reduced by NADH) reacted with O₂ followed at 450 nm and 540 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm and 540 nm over time. *Concentration after mixing.

Wavelength (nm)	[O ₂] (μM)*	k' ₁ (s ⁻¹)	k' ₂ (s ⁻¹)
450	130	121.4 ± 26.9	3.2 ± 2.8
540	130	-----	6.7 ± 2.2

4.2 Class B FDP from *Escherichia coli*, FDP core

4.2.1 Biochemical characterization of *E. coli* FDP core

Both FDP core (FIRd lacking the rubredoxin domain) and the rubredoxin domain were overexpressed in *E. coli* BL21(DE3) Gold and purified based on the procedure previously described by Gomes and co-workers [19]. After the purification steps, the purity of the resulting fractions obtained was assessed by UV-Visible spectroscopy and SDS-PAGE as illustrated in Figure 20 A and B.

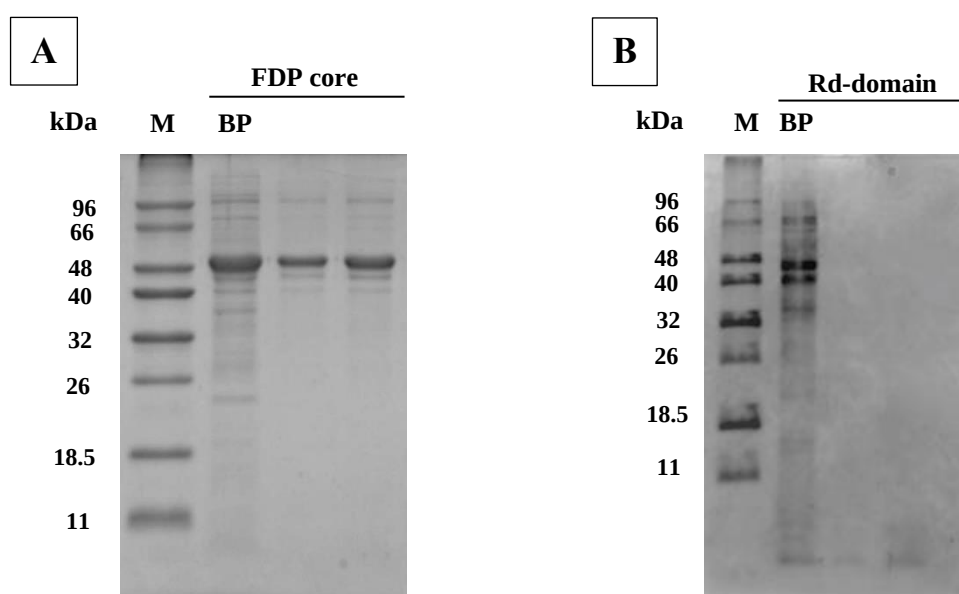


Figure 20: SDS-PAGE analysis of the purity of the fractions FDP core and Rd-domain after the purification steps. Panel A- The second lane corresponds to the protein before purification (BP), while the third and fourth lanes correspond to two different amounts of the final sample after purification, 0.5 µg and 0.9 µg samples. Panel B- The second lane corresponds to the protein before purification (BP), while the third and fourth lanes correspond to two different amounts of the final sample after purification, 0.2 µg and 0.4 µg samples. In both gels, the first lane (M) corresponds to the molecular masses markers (NZY protein marker II).

As predicted by the amino acid sequence of the two proteins, the gels present intense bands at around ~48 kDa, Figure 20 A, and ~6 kDa, Figure 20 B, which correspond to the FDP core and rubredoxin domain, respectively. However, the presence of thinner and less intense bands, in Figure 20 A, indicates that the protein is not completely pure, i.e., there are vestigial amounts of contaminant proteins in the FDP core sample. On the contrary, the gel in Figure 20 B, does not

present other bands than the one for the desired protein, indicating that the rubredoxin-domain sample is close to the purity.

As for FIRD, total protein concentration was determined by the BCA method, yielding values of 410 μ M for the FDP core and 16 μ M for the rubredoxin-domain. The iron content of the FDP core was determined by the orto-phenantroline method [27] [52] yielding 1.61 ± 0.01 Fe/monomer. This value is lower than the expected two atoms per diiron center. As for FIRD, this result suggests that complete iron incorporation was not achieved despite the iron supplementation in the growth medium. Based on the literature, this partial incorporation of iron is common in other FDPs overproduced in *E. coli*. [53]

The flavin content was determined by UV-Visible determined as 0.63 ± 0.02 FMN/monomer. This value showed that full flavin incorporation of one flavin cofactor in the flavodoxin-like domain was also not obtained. Since the FMN is non-covalently bound to the protein, the whole purification process from cell lysis until protein concentration may lead to the loss of this cofactor. However, this FMN content is in-line when compared with what is found in the literature for other FDPs overproduced in *E. coli*. [53]

The UV-visible spectra of the *E. coli* FDP core in the as isolated state contains only the contribution of the FMN with absorption maxima at 370 and 460 nm as shown in Figure 21.

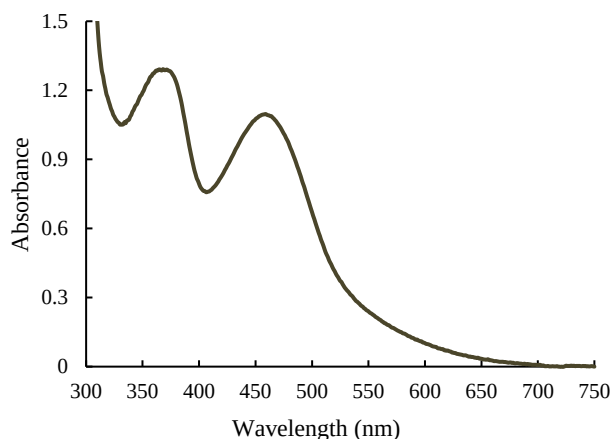


Figure 21: UV-Visible spectra of FDP core. The black solid line represents the FDP core in the fully oxidized state. Concentrations: [FDP core]= 30 μ M, in 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

4.2.2 Optimization of the *E. coli* FDP core-NO binding conditions

The FDP core incubation with NADH was monitored by UV-Visible spectroscopy and allowed us to deconvolute the differential reduction of the FMN.

The protein sample was incubated with different amounts of NADH (in the presence of 5 μ M FlRd reductase and 5 μ M rubredoxin domain) and the spectrum after each addition was recorded, as shown in Figure 22. After addition of 1.5 eq. of NADH a total disappearance of the visible component of the spectrum, Figure 22, is observed, indicating that the FMN was totally reduced. Although 2 eq. of NADH were expected to reduce completely the FDP core, the fact that the FMN and iron incorporation were not complete explains why this is achieved with a smaller amount of reductant. In fact, after the incubation with 1.5 eq. of NADH, a slight absorption band is observed with maximum at ~ 340 nm, which is indicative of a small excess of NADH.[54]

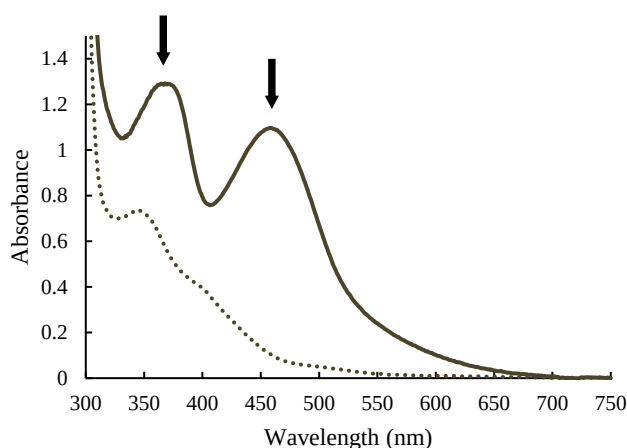


Figure 22: FDP core reduction by NADH. The black solid line represents the as-purified FDP core spectrum. The black dotted line represents the spectrum of the protein incubated with 1.5 eq. of NADH. Arrows point to the absorbance changes at different wavelengths. Concentrations: [FDP core] = 150 μ M; [Rd-domain] = 5 μ M; [Reductase] = 5 μ M, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

After determining the proper conditions to obtain a fully reduced FDP core, similarly as before and as shown in Figure 22 (black line) without excess of NADH, the oxidation with its main substrate, NO, was monitored by the incubation with different amounts, Figure 23. Also, the samples represented in Figure 23 are the ones that were monitored by EPR (see next Section).

The incubation with 0.5 eq. of NO leads to the formation of the semiquinone form of FMN, observed by the absorption bands at 360 nm and at 490 nm. The addition of 1.5 eq. of NO, leads to the total FMN oxidation, indicating that the protein is in its fully oxidized state. This is observed by the increase in the absorption bands at 360 nm and 460 nm, revealing a final spectrum similar to the as-purified protein spectrum, as shown in Figure 23 A.

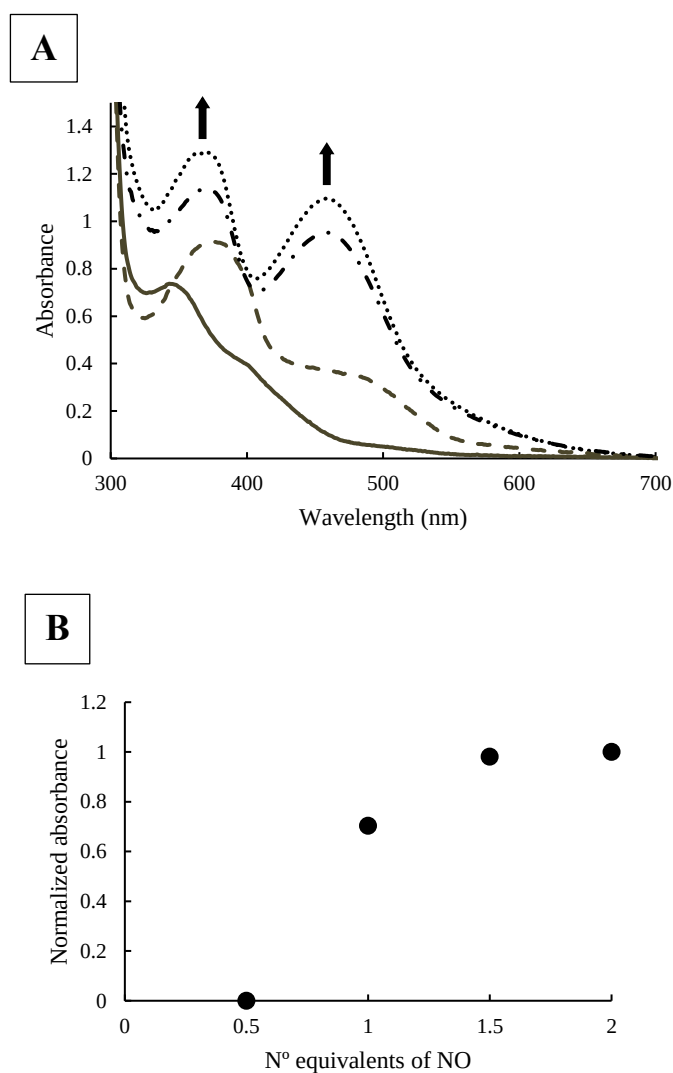


Figure 23: FDP core re-oxidation by NO. Panel A- The black dotted line represents the FDP core in the as-isolated state; the black solid line represents the spectrum after incubation with 1.5 eq. of NADH; the black dashed line represents the spectrum after the incubation with 0.5 eq. of NO and the black dashed and dotted line represents the spectrum after incubation with 2 eq. of NO. Arrows point to the absorbance changes at different wavelengths. Panel B- The black points represent the normalized absorbance at 460 nm for each addition of NO. Concentrations: [FDP core]= 150 μ M; [Rd-domain]= 5 μ M; [Reductase]= 5 μ M, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

4.2.3 *E. coli* FDP core-NO binding monitored by EPR

After determining the best conditions to study the FDP core-NO binding, the samples that were studied before by UV-Visible spectroscopy were now monitored by EPR spectroscopy, Figure 24. The EPR spectrum of FDP core incubated with 1.5 eq. of NADH revealed, as expected, no signal related to the diiron site, which is indicative of the $\text{Fe}^{\text{II}}\text{-Fe}^{\text{II}}$ state that because of the antiferromagnetically coupling, gives rise to system with a total spin $S = 0$. No features of the diiron site in the mixed valence state $\text{Fe}^{\text{III}}\text{-Fe}^{\text{II}}$ were observed during the experiment. Combining these results with the UV-visible spectra of these samples, this may indicate that the NO was always converted into N_2O and the diiron center went directly from the fully reduced to the fully oxidized state. Furthermore, the incubation with different amounts of NO gave rise to an axial signal with g values at 2.04 and 2.02. This signal is typical of iron-dinitrosyl complexes: The signal at $g=2.0$ is due to the semiquinone form of the FMN.

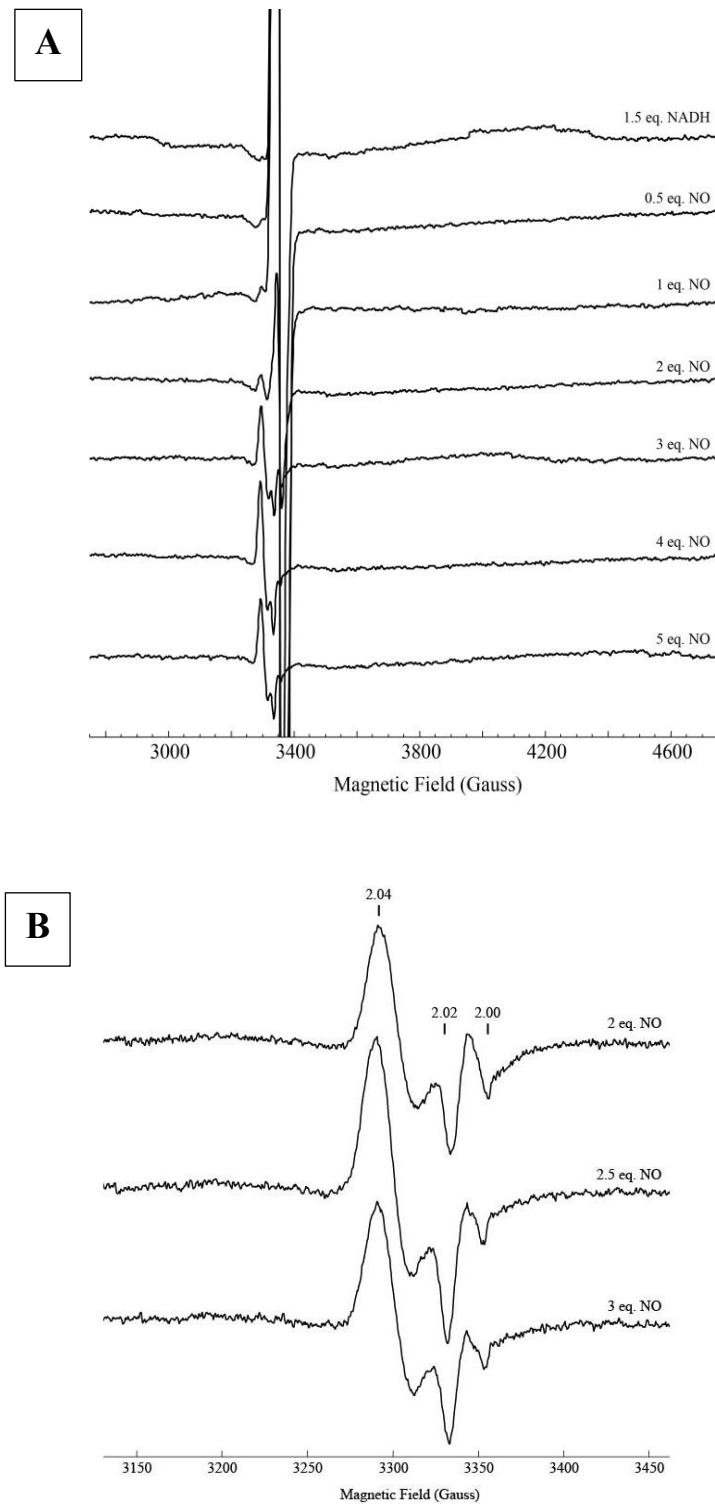


Figure 24: EPR spectra of FDP core incubated with NO. Panel A- EPR spectra of FDP core after reduction with 1.5 eq. of NADH and different amounts of NO (0.5 eq. until 5 eq. NO). Panel B- EPR spectra of the same samples as described above collected in a spectral window centered in the g values predicted for the diiron center signal. Concentrations: [FDP core] = 150 μ M, [Rd-domain] = 5 μ M; [Reductase] = 5 μ M, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol. The spectra were collected at T= 7 K; microwave power 2 mW and microwave frequency= 9.39 GHz.

4.2.4 Study of the reaction of *E. coli* FDP core with NO by stopped-flow spectroscopy

The reaction of the *E. coli* FDP core with NO was also investigated by stopped-flow spectroscopy under anaerobic conditions at 15°C. The goal was to determine the rate constants attributable to the FMN oxidation, without the spectral contribution of the rubredoxin center. The protein was previously reduced with a stoichiometric amount of NADH in order not to have NADH excess, similarly as before and as shown in Figure 23 A (black line). Upon mixing of protein with a NO-saturated buffer, the spectral changes of the FDP core were monitored. As before, the assays were monitored in two timescales, 0.45 s and 45 s, were used in order to calculate the different reaction rates. However, in the first spectrum, recorded at 3.7 ms, already shows that the reaction with NO led to a pronounced absorbance band at 450 nm, corresponding to the oxidized FMN. The absorbance of this band only increases slightly in the 45 s spectrum, Figure 25. This indicates that the reaction is faster than the timescale of the experiments.

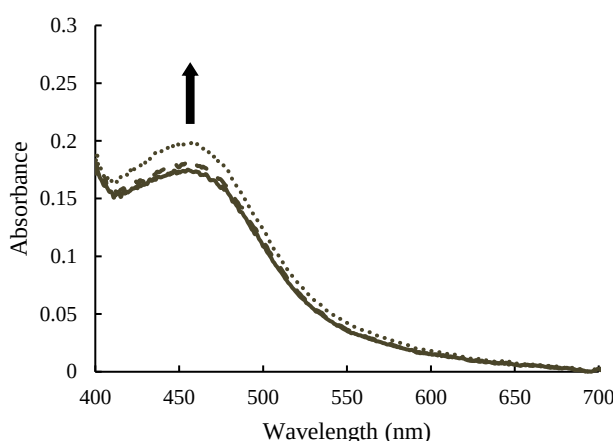


Figure 25: Reaction of FDP core (reduced by NADH) with NO followed by stopped-flow spectroscopy. The black solid line represents the first spectrum collected at 3.7 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for analysis. Concentrations after mixing: [FDP core]= 30 μ M; [Rd-domain] = 0.5 μ M; [Reductase]= 0.5 μ M; [NO] = 955 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

The excess of NO allowed us to apply a pseudo-first order kinetic approach. Thus, the absorbance was monitored over time at 450 nm, as shown in Figure 26. As depicted in Figure 26, the addition of NO led to a slight increase in absorbance at 450 nm during the first 8 s, Figure 26 B. In the remaining time up to the 45 s, no relevant changes were observed, Figure 26 B. Again, the data was fitted with one exponential function, and respective rate constants are summarized below in

Table 4. Because the FMN co-factor is already almost completely oxidized state in the first spectrum, which indicates that the reaction occurs mainly during the deadtime of the experiment, the rate constant calculated, $k'_2 = 10.6 \text{ s}^{-1}$ may be significantly underestimated.

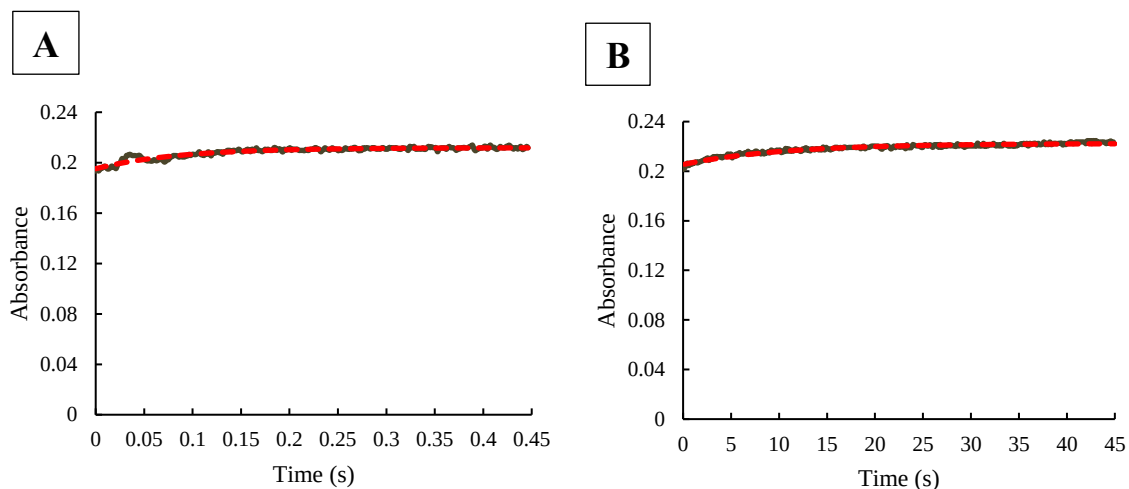


Figure 26: Typical traces and fits at 450 nm of FDP core (reduced by NADH) reacted with NO followed by stopped-flow spectroscopy. Panel A- The black solid line represents the variation in the absorbance at 450 nm in the 0.45 s window assay. The fit to the curve is represented by the red dashed line. Panel B- The black solid line represents the variation in the absorbance at 450 nm in the 45 s window assay. The fit to the curve is represented by the red dashed line. Concentrations after mixing [FDP core]= 30 μM ; [Rd-domain]= 0.5 μM ; [Reductase]= 0.5 μM ; [O_2]= 130 μM ; $T = 15^\circ\text{C}$, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 4: Calculated reaction rates of the FDP core (reduced by NADH) reacted with NO and followed at 450 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm over time. *Concentration after mixing.

[NO] (μM)*	$k'_1 \text{ (s}^{-1}\text{)}$	$k'_2 \text{ (s}^{-1}\text{)}$
955	-----	10.6 ± 1.65

4.2.5 Study of the reaction of *E. coli* FDP core with O_2 by stopped-flow spectroscopy

The reaction of the FDP core, previously reduced anaerobically with NADH and as shown in Figure 14 A (black line), was incubated with O_2 by mixing the reduced protein with air-equilibrated buffer ($\sim 260 \mu\text{M O}_2$) in a 1:1 ratio. Upon mixing with O_2 , the first spectrum acquired at 0.75 ms, contrary to the reaction with NO, does not exhibit any spectral changes from the

reduced FDP core. At 0.45 s the spectrum, corresponding to an almost fully oxidized FMN, is observed, with a maximum absorbance at 450 nm. The spectrum changes slightly up to 45 s, as shown in Figure 27.

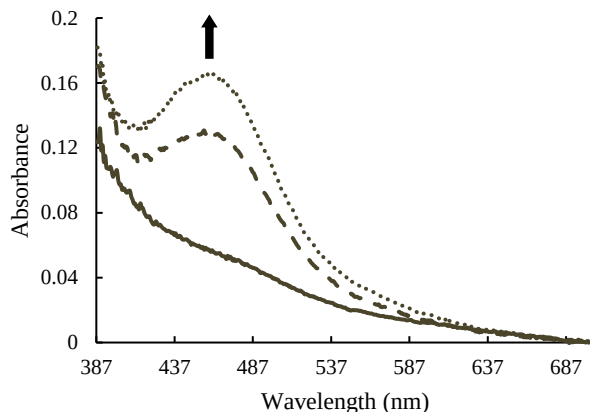


Figure 27: Reaction of FDP core (reduced by NADH) with O₂ followed by stopped-flow spectroscopy. The black solid line represents the first spectrum collected at 0.75 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for the analysis. Concentrations: [FDP core] = 30 μ M; [Rd-domain]= 0.5 μ M; [Reductase]= 0.5 μ M; [O₂]=130 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

As before, the absorbance was monitored over time at 450 nm, as shown in Figure 28. During this reaction, where the pseudo-first order kinetic conditions were maintained, two different processes were observed, leading to the fitting of the experimental data with a sum of two exponential decays, as summarized in equation 7. The obtained rate constants are summarized below in Table 5.

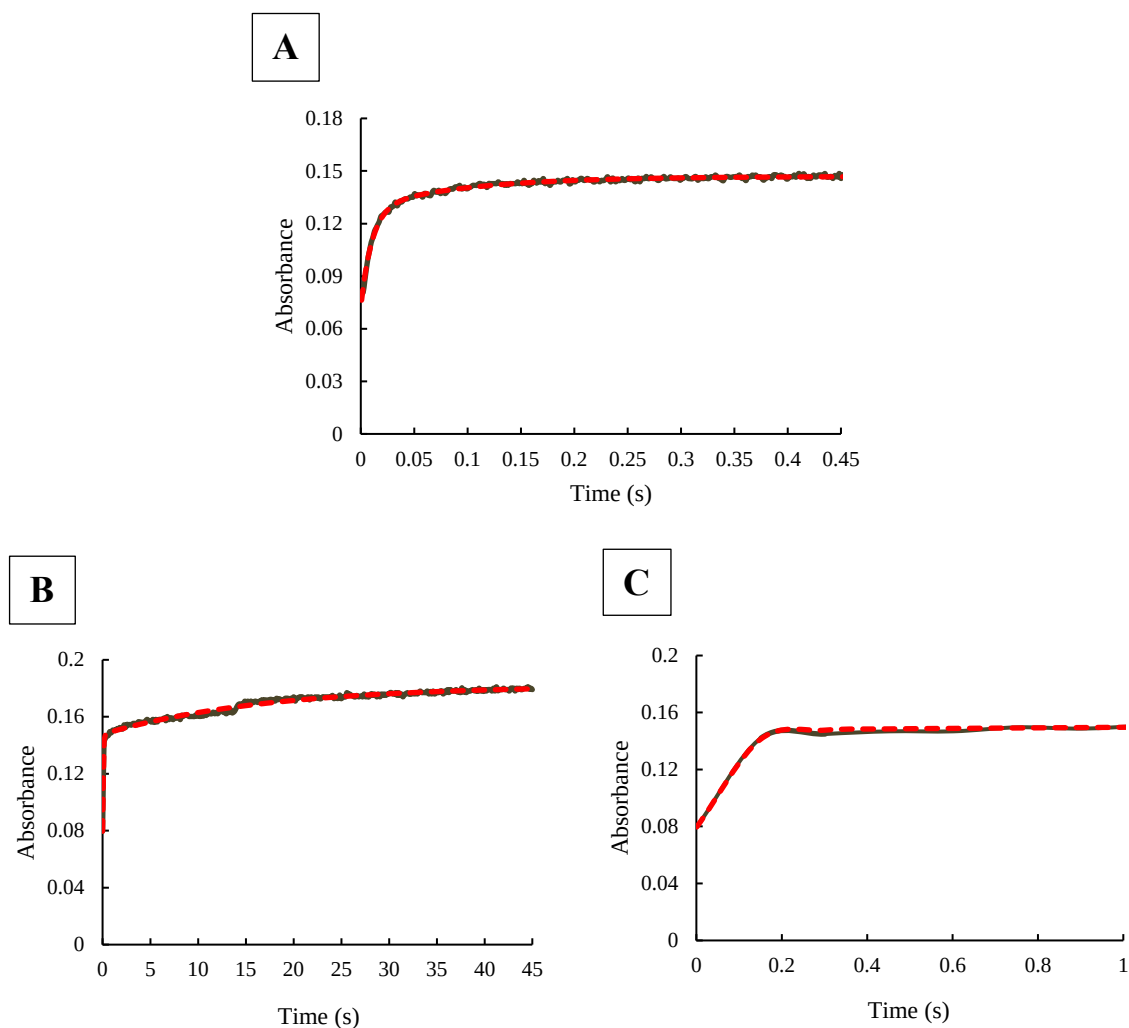


Figure 28: Typical traces and fits at 450 nm of FDP core (reduced by NADH) reacted with O₂ followed by stopped-flow spectroscopy. Panel A- The black solid line represents the variation in the absorbance at 450 nm in the 0.45 s window assay. The fit of the curve is represented by the red dashed line. Panel B- The black solid line represents the variation in the absorbance at 450 nm in the 45 s window assay. The fit of the curve is represented by the red dashed line. Panel C- An amplification of panel B up to 1 s. Concentrations after mixing [FDP core]= 30 μ M; [Rd-domain]= 0.5 μ M; [Reductase]= 0.5 μ M and [O₂]= 130 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 5: Calculated reaction rates of the FDP core (reduced by NADH) reacted with O₂ and followed at 450 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm over time. * Concentration after mixing.

[O ₂] (μ M)*	k' ₁ (s ⁻¹)	k' ₂ (s ⁻¹)
130	97.2 $\pm$ 1.9	9.6 $\pm$ 1.6

4.3 Class F FDP from *Clostridioides difficile*

4.3.1 Biochemical characterization of *C. difficile* FDP_F

Clostridioides difficile has two different FDPs. One of these FDPs is a member of class F and will be designated as FDP_F. FDP_F was overexpressed in *E. coli* BL21(DE3) Gold and purified based on Folgosa and co-workers [22]. After the purification steps, the purity of the resulting fractions was assessed by UV-Visible spectroscopy and SDS-PAGE as illustrated in Figure 29.

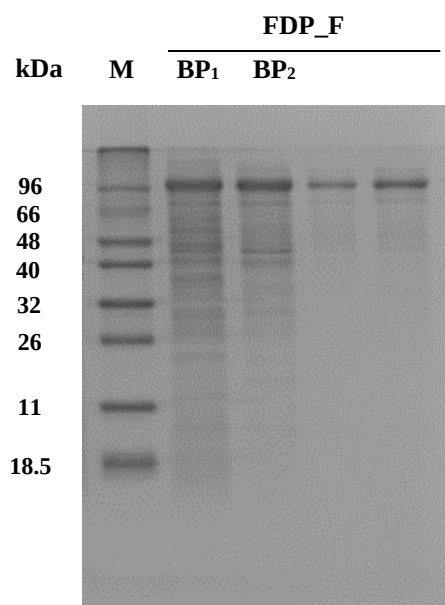


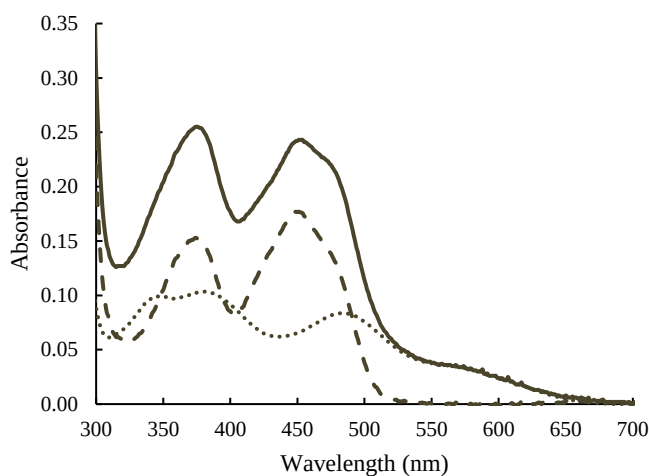
Figure 29: SDS-PAGE analysis of the purity of the FDP_F fractions after the purification steps. The second and third lanes correspond to the cell extract before purification (BP). The fourth and fifth lanes correspond to two different amounts of FDP_F after purification, 1.9 μ g and 3.8 μ g samples. In the gel, the first lane (M) corresponds to the molecular mass markers (NZY protein marker II).

The SDS-PAGE presented in Figure 29 shows, as expected, in the lane correspondent to the *E. coli* extract, a variety of bands corresponding to all the proteins expressed. The molecular mass of FDP_F is 97 kDa, which corresponds to the most intense band observed in the SDS-PAGE, indicating that the protein was successfully overexpressed. However, the presence of less intense bands suggests that the protein is not completely pure.

The protein concentration of the final fraction was calculated by the BCA method, being obtained a value of 81 μ M FDP_F. The iron content of the FDP_F sample was determined by the ortho-phenantroline method [27][52], yielding a value of 1.33 ± 0.00 Fe/monomer. This value is lower than expected of three atoms, two atoms for the diiron site and one atom for the rubredoxin

domain. This value suggests that complete iron incorporation was not achieved, despite the iron supplementation in the growth medium. However, compared to previously reported studies, this partial incorporation of iron is common in similar proteins overproduced in *E. coli* [22].

The UV-Visible spectrum of FDP_F in the as isolated state shows the characteristic features for the flavin co-factors and the rubredoxin center. The spectrum of the FDP_F exhibits peaks with maxima of absorbance at 384 and 450 nm, typical for the oxidized flavin co-factors, and a broader band at 570 nm, resulting from the rubredoxin center contribution, as shown in Figure 30.



4.3.2 Optimization of the *C. difficile* FDP_F-NO binding conditions

Figure 30: UV-Visible spectra of FDP_F. The black solid line represents the FDP_F spectrum in the fully oxidized state, while the black dashed line and black dotted line represent the rubredoxin and flavin contributions, respectively. The flavin spectrum results from the subtraction of the rubredoxin spectrum contribution from the FDP_F spectrum. Concentrations: [FDP_F]= 25 μ M; [Rd-domain] = 15 μ M, in 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

As for the other FDPs under study, the reduction conditions were also studied for FDP_F. Again, the sample was incubated with sub-stoichiometric amounts of NADH, and the spectrum after each addition was acquired, as shown in Figure 31. The spectrum of FDP_F in the as isolated form presents peaks at 384 and 450 nm, and a broader band at 570 nm. As expected, after the incubation with 3.5 eq. of NADH, a total bleaching of these absorption bands in the visible region of the spectrum was observed, indicating the complete reduction of the protein's co-factor. Concomitantly, a broad band with a maximum at $\sim$ 690 nm was formed, corresponding to the formation of a charge transfer complex between NAD^+ and the reduced FAD.[22]

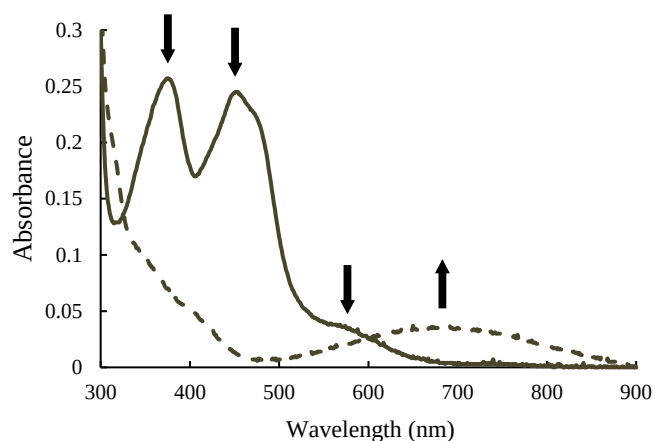


Figure 31: FDP_F reduction by NADH. The black solid line represents the spectrum of the as isolated (oxidized) FDP_F. The black dashed line represents the spectrum of the protein after incubation with 3.5 eq. of NADH. Arrows point to the observed absorbance changes at different wavelengths. Concentrations: [FDP_F] = 25 μ M and Buffer 50mM Tris-HCl pH 7.5 containing 18% glycerol.

As the main goal was to utilize FDP_F to monitor the binding of NO, knowing that it has a quite low reaction rate with this substrate [22], incubation with different amounts of NO was performed and the UV-Visible spectrum was recorded after each addition. The protein was first fully reduced by the addition of 3.5 eq. of NADH, similarly as before and as shown in Figure 32 A (black line). After reduction, the oxidation of the FDP_F was performed by adding increasing amounts of NO, Figure 32. After incubation with 3 eq. of NO, a small amount of oxidized flavin co-factor is observed by the increase in the absorbance at 384 and 450 nm. Concomitantly, the intensity of the charge transfer band is reduced. The addition of 6 eq. of NO leads to a fully oxidized FDP_F with a final spectrum similar to that of the as purified FDP_F, as shown in Figure 32 A.

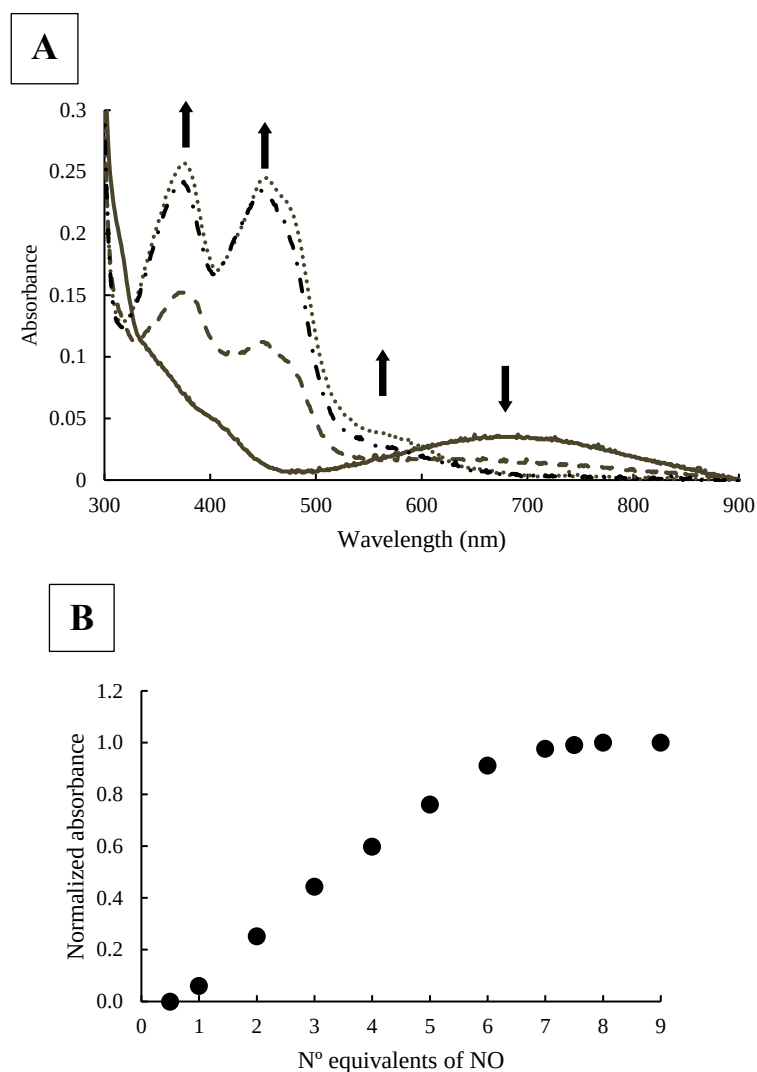


Figure 32: FDP_F re-oxidation by NO. Panel A- The black dotted line represents FDP_F in the as isolated form. The black solid line represents FDP_F after incubation with 3 eq. of NADH. The black dashed line represents the spectrum of FDP_F after incubation with 3 eq. of NO. The black dashed and dotted line represents the spectrum of FDP_F after incubation with 9 eq. of NO. Arrows point to the absorbance changes at different wavelengths. Panel B- The filled circles represent the normalized absorbance at 460 nm after each sub-stoichiometric addition of NO. Concentrations: [FDP_F] = 25 μ M and Buffer 50mM Tris-HCl pH 7.5 containing 18% glycerol.

4.3.3 *C. difficile* FDP_F-NO binding monitored by EPR

In order to study the NO binding to the diiron catalytic site of FDP_F, similar samples to the ones used in the UV-visible experiments were prepared and monitored by EPR spectroscopy, Figure 33. The EPR spectrum of FDP_F incubated with 4 eq. NADH revealed, as expected, no signal for the diiron site or rubredoxin center. The incubation of the reduced sample with 2 eq. NO generates an EPR spectrum with a resonance at a g-value of 4.28, that increases with the NO amount, giving

rise also to a minor resonance at a g-value of 9.19, that is consistent with the contribution of the rubredoxin center in the oxidized state. These signals are attributed to the middle $|\pm 3/2\rangle$ and lower $|\pm 1/2\rangle$ Kramer doublets of a high spin ferric center with $S=5/2$. The addition of the 2 eq. of NO also gives rise to a quasi-axial signal with g-values of 1.95, 1.82 and 1.80, that is consistent with the catalytic diiron site in a mixed valence state ($\text{Fe}^{\text{III}}\text{-Fe}^{\text{II}}$), with a total spin system of $S=1/2$. As shown in Figure 33 B, the intensity of this signal increases until the incubation with 5 eq. of NO and then remains unaltered. Furthermore, the incubation with different amounts of NO gave rise to an axial signal with g values at 2.04. This signal is typical of iron-mononitrosyl complexes. The intense signal at $g=2.0$ is due to the semiquinone form of the FMN.

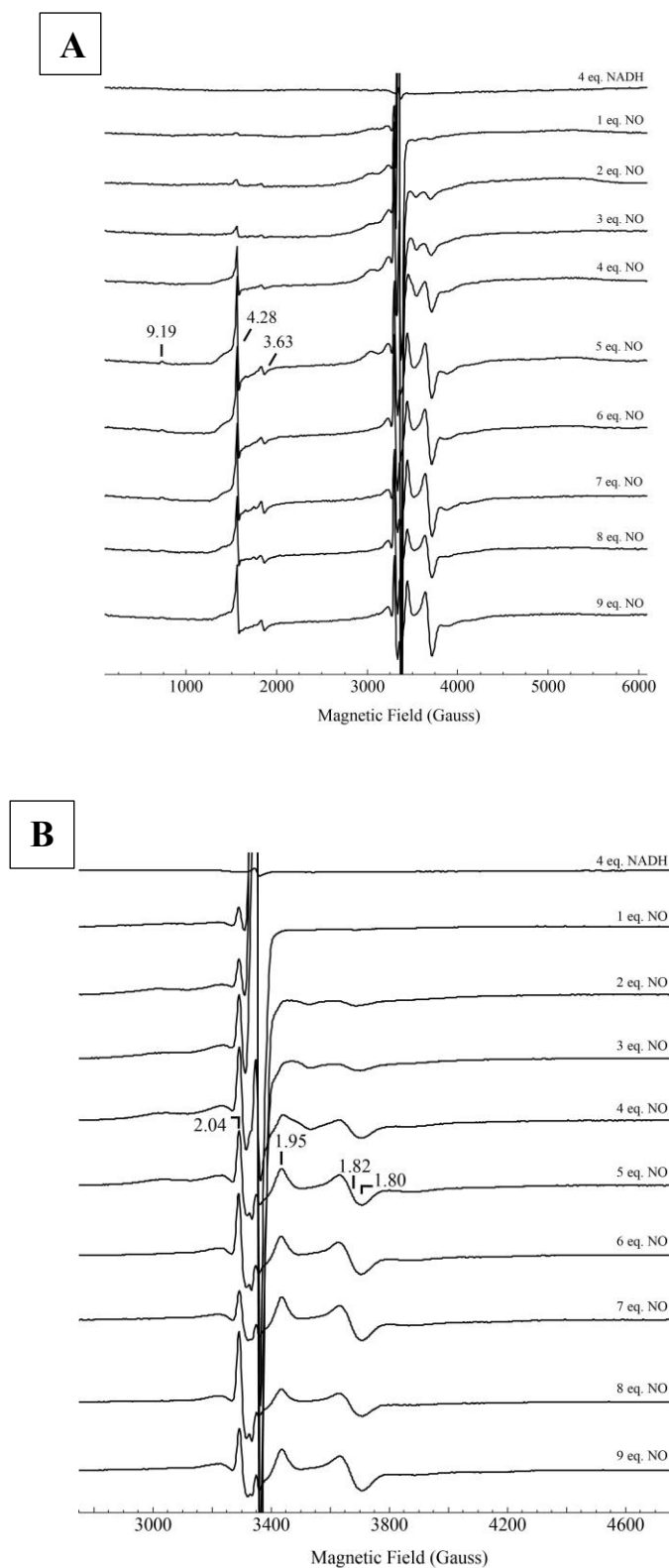


Figure 33: EPR spectra of FDP_F incubated with NO. Panel A- EPR spectra of the FDP_F after reduction with 4 eq. of NADH and addition of different stoichiometric amounts of NO (1 eq. until 9 eq. NO). Panel B- EPR spectra of the same samples as described above collected in a spectral window centered in the diiron center signal. Arrows indicate the g values assigned to each signal. Concentrations: [FDP_F] = 83 μ M and Buffer 50mM Tris-HCl pH 7.5 containing 18% glycerol. The spectra were collected at T = 7K; microwave power 2 mW and microwave frequency= 9.39 GHz

Besides the EPR signals described above, in the FDP_F sample incubated with 5 eq. of NO it was also possible to identify other signals with g values of 2.22, 2.08 and 1.73. Therefore, to properly identify the signals, EPR spectra were recorded at different temperatures (7, 11, 20, 30, and 40 K). This experiment allowed us to determine that the signals with $g = 2.22$ and 1.73 were part of the same species, as their intensity changes in the same way with the temperature, as shown in Figure 34. On the other hand, the signal at $g = 2.08$ did not change in the same way, which indicates that is due to a different species. Most likely it is equivalent to the signals at $g = 2.04$ that were observed in the *E. coli* F1Rd and FDP core samples. As expected, the intensity of the signals related to the diiron center also changed with the temperature, being the maximum at 11K and being observable until 30 K.

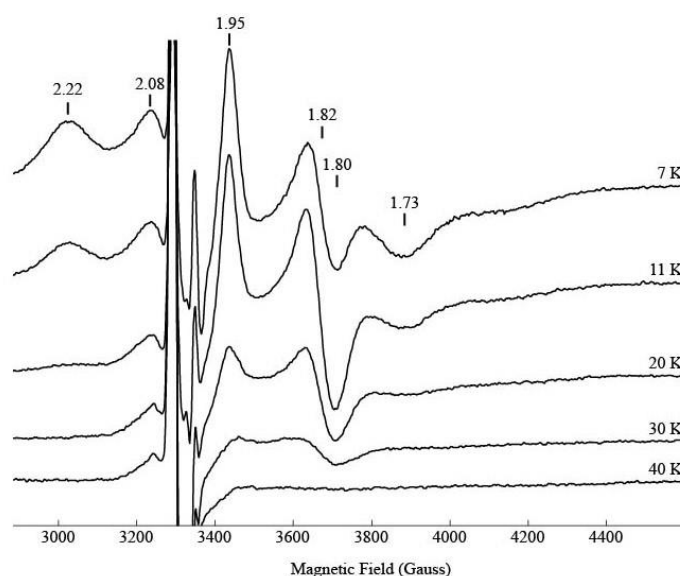


Figure 34: EPR spectra of FDP_F recorded at different temperatures. The spectra were acquired with the sample incubated with 5 eq. of NO. Arrows indicate the g values assigned to each signal. Concentrations: [FDP_F] = 83 μ M and Buffer 50mM Tris-HCl pH 7.5 containing 18% glycerol. The spectra were collected at T= 7, 11, 20, 30 and 40 K; microwave power 2 mW and microwave frequency= 9.39 GHz.

4.3.4 Study of the reaction of *C. difficile* FDP_F with NO by stopped-flow spectroscopy

The reaction of the FDP_F with NO was also investigated by stopped-flow spectroscopy under anaerobic conditions at 15°C. As for the other FDPs, the protein was previously reduced in anaerobic conditions with stoichiometric amounts of NADH, similarly as before and as shown in Figure 32 A (black line). Upon reaction with NO, the first spectrum acquired at 0.75 ms is very

similar to the fully reduced protein, presenting a broad band with a maximum at 630 nm, characteristic of the charge transfer complex of FADH₂ with NAD⁺. The spectrum at 0.45 s, shows a broad band at 450 nm, indicative of a partial oxidation of one of the flavin co-factors. The spectrum at 45 s shows an increased absorption of the band with the maximum at 450 nm, indicative of the fully oxidation of the two flavin co-factors. The reoxidation of the rubredoxin center is not clear in this experiment, Figure 35.

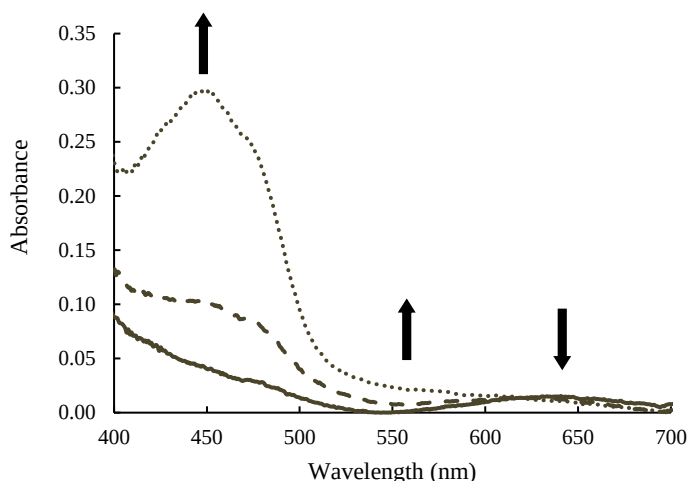


Figure 35: Reaction of FDP_F (reduced by NADH) with NO followed by stopped-flow spectroscopy. The black solid line represents the first spectrum collected at 0.75 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for the analysis. Concentrations after mixing [FDP_F]= 30 μ M; [NO]= 955 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

The excess of NO allowed us to apply a pseudo-first order kinetic approach. Thus, the absorbance was monitored at 450 nm over time, with two timescales, 0.45 s and 45 s, as shown in Figure 36. Addition of NO led to an increase in absorbance at 450 nm during the 45 s of the assay, with a faster process in the first 7 s of the reaction, Figure 36 A and B. Therefore, the reaction rate was calculated by fitting with one exponential function, and the respective rate constant is summarized below in Table 6.

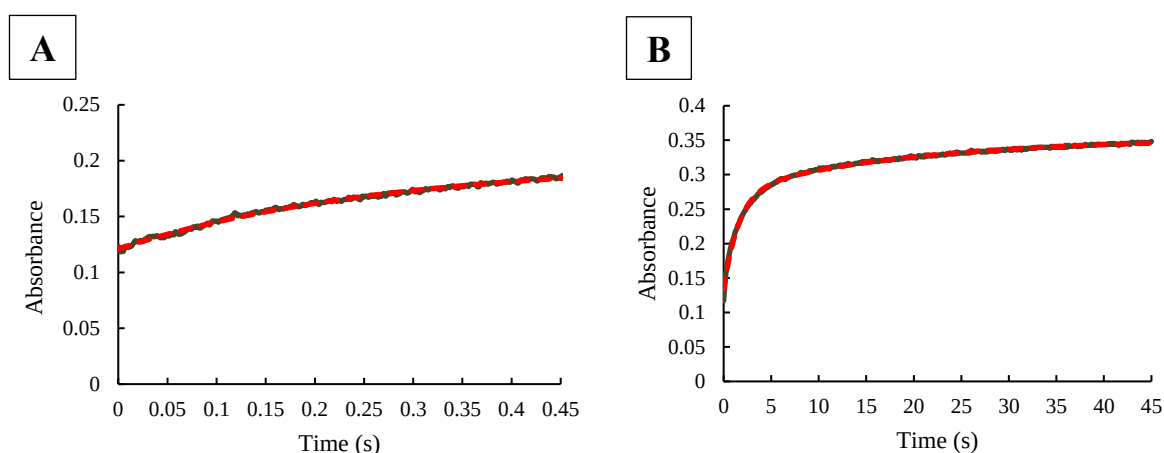


Figure 36: Typical traces and fits at 450 nm of FDP_F (reduced by NADH) reacted with NO followed by stopped-flow spectroscopy. Panel A- The black line represents the variation in the absorbance at 450 nm in the 0.45 s window assay. The fit to the curve is represented by the red dashed line. Panel B- The black line represents the variation in the absorbance at 450 nm in the 45 s window assay. The fit to the curve is represented by the red dashed line. Concentrations after mixing [FDP_F]= 30 μ M; [NO]= 955 μ M, T= 15°C, in 50 mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 6: Calculated reaction rates of the FDP_F (reduced by NADH) reacted with NO and followed at 450 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm over time. *Concentration after mixing.

[NO] (μ M)*	k'_1 (s^{-1})	k'_2 (s^{-1})
955	-----	4.0 ± 0.9

To avoid the formation of the charge transfer complex with NAD^+ and its contribution to the spectrum, FDP_F was anaerobically reduced with stoichiometric amounts of NADPH and the reaction with NO was then monitored as before by mixing of the reduced protein with NO saturated buffer. Upon reaction with NO, the spectral evolution was similar to the previously observed when NADH was used as reductant, Figure 37, leading to a complete oxidation of the flavin co-factor of the protein. Again, the reoxidation of the rubredoxin was not observed in the timescale of the experiment.

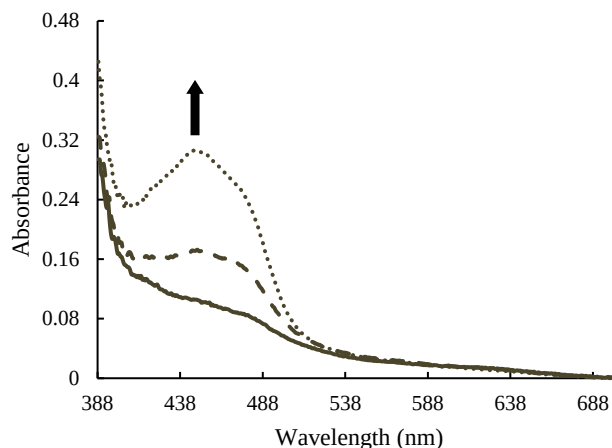


Figure 37: Reaction of FDP_F (reduced by NADPH) with NO followed by stopped-flow spectroscopy. The black solid line represents the first spectrum collected at 2.26 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for the analysis. Concentrations after mixing [FDP_F]= 30 μ M; [NO]= 955 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

The excess of NO allowed us to apply a pseudo-first order kinetic approach. Thus, the absorbance was monitored over time at 450 nm, as shown in Figure 38. Addition of NO led to an increase in absorbance at 450 nm during the first 18 s of the reaction, with a faster process in the first 8 s of the reaction, Figure 38 A and B. In this case, we were able to determine the reaction rate for the faster process. Therefore, in this case the reaction rates were calculated by fitting a sum of two exponential functions to the data as in equation 7, are summarized below in Table 7.

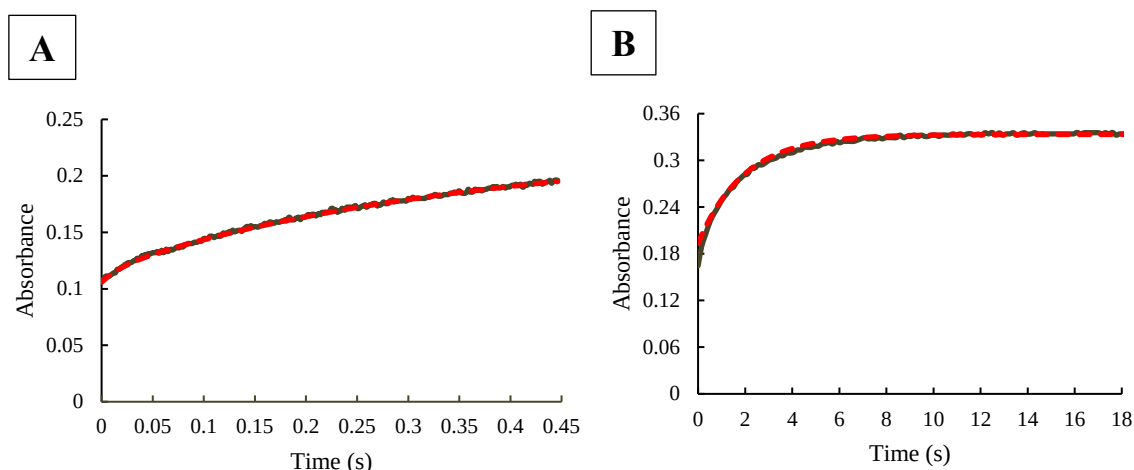


Figure 38: Typical traces and fits at 450 nm of FDP_F (reduced by NADPH) reacted with NO and followed by stopped-flow spectroscopy. Panel A- The black line represents the variation in the absorbance at 450 nm in the 0.45 s window assay. The fit to the curve is represented by the red dashed line. Panel B- The black line represents the variation in the absorbance at 450 nm in the 18 s window assay. The fit to the curve is represented by the red dashed line until 19 s. Concentrations after mixing [FDP_F]= 30 μ M; [NO]= 955 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 7: Calculated reaction rate of the FDP_F (reduced by NADPH) reacted with NO and followed at 450 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm over time. *Concentration after mixing.

[NO] (μ M)*	k_1 (s^{-1})	k_2 (s^{-1})
955	50.5 ± 13.7	2.6 ± 0.2

As for FIRD, in both experiments with FDP_F (reduced with NADH or NADPH), the oxidation of the rubredoxin center was not observed, indicating that also with this enzyme, the reaction with excess of NO in single turnover conditions may lead to damage of the protein, namely its rubredoxin center.

4.3.5 Study of the reaction of *C. difficile* FDP_F with O₂ by stopped-flow spectroscopy

The *C. difficile* FDP_F was previously reduced in anaerobic conditions with a stoichiometric amount of NADH and then reduced FDP_F was mixed with air-equilibrated buffer (~260 μ M O₂) in a 1:1 ratio. The first spectrum, recorded at 3.7 ms exhibits a broad band with a maximum at 450 nm, indicative of a partially oxidized flavin co-factor. On the other hand, the charge transfer

complex is not observed, indicating that its disappearance occurs in the dead time of the instrument, Figure 39.

A significant increase in absorbance at 450 nm and 570 nm is then observed until the 0.45 s, corresponding to the oxidized rubredoxin center. In the spectrum recorded at 45 s, the fully oxidized FDP_F is observed.

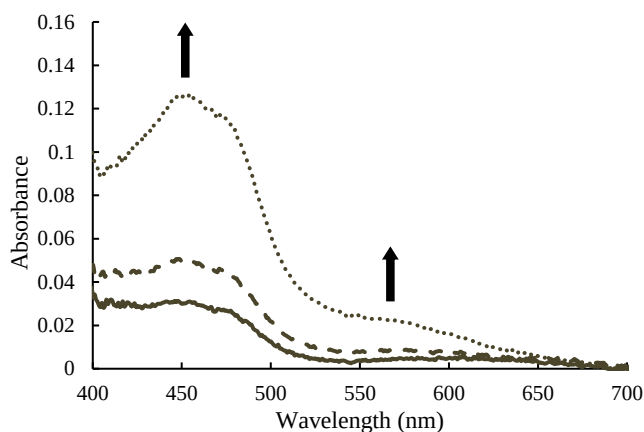


Figure 39: Reaction of FDP_F (reduced by NADH) with O₂ followed by stopped-flow spectroscopy. The black solid line represents the first spectrum collected at 3.7 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for analysis. Concentrations after mixing [FDP_F]= 30 μ M; [O₂]= 130 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Like in the reaction with NO, the experimental conditions used allowed to apply a pseudo-first order kinetic approach. The analysis was monitored at 450 nm over time, as shown in Figure 40. Addition of substrate led to an increase in absorbance at 450 nm during the 45 s, with a faster process in the first 20 s of the reaction, Figure 40 A and B. Therefore, the reaction rates were calculated by fitting of one exponential function, and respective rate constants are summarized below in Table 8.

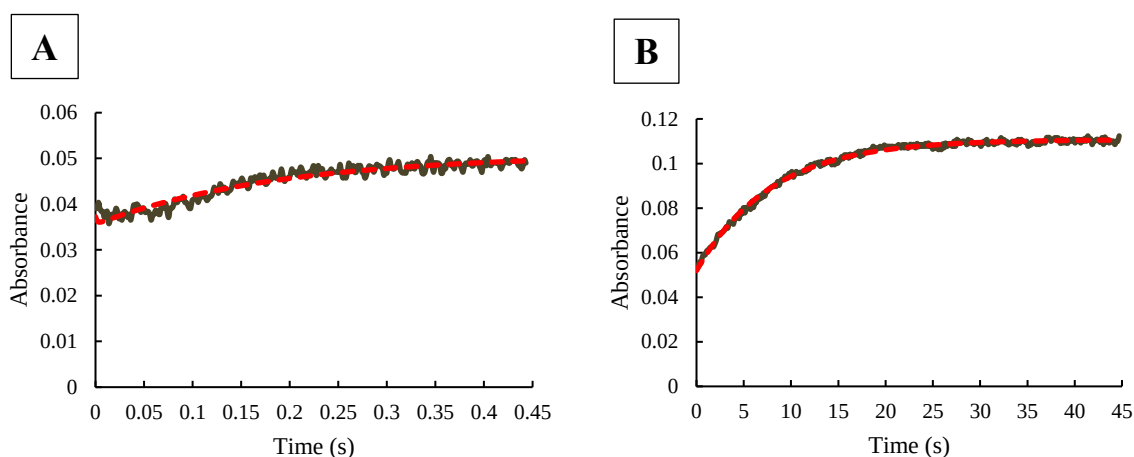


Figure 40: Typical traces and fits at 450 nm of FDP_F (reduced by NADH) reacted with O₂ and followed by stopped-flow spectroscopy. Panel A- The black line represents the variation absorbance at 450 nm in the 0.45 s window assay. The fit to the curve is represented by the red dashed line. Panel B- The black line represents the absorbance variation at 450 nm in the 45 s window assay. The fit to the curve is represented by the red dashed line. Concentrations after mixing [FDP_F]= 30 μ M; [O₂]= 130 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 8: Calculated reaction rate of the FDP_F (reduced by NADH) reacted with O₂ and followed at 450 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm over time. *Concentration after mixing.

[O ₂] (μ M)*	k' ₁ (s ⁻¹)	k' ₂ (s ⁻¹)
130	-----	4.6 $\pm$ 0.6

Similar experiments were performed but using NADPH as reducing agent to avoid the formation of the charge transfer complex. Upon reaction with 260 μ M O₂ (air-equilibrated buffer) the spectral evolution observed was similar to the previous one when NADH was used as reductant, Figure 41. However, in this case, the first spectrum, recorded at 6.95 ms already shows partially oxidized flavin and rubredoxin center, supported by the absorbance bands at 450 nm and 570 nm. This result indicates that the first reaction step is faster than the dead time of the experiments. The development of the reaction leads to the increase in the absorbance of these bands until the 45 s, Figure 41.

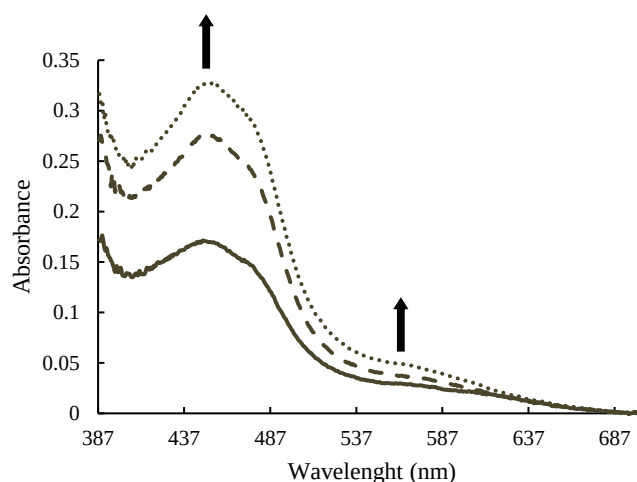


Figure 41: Reaction of FDP_F (reduced by NADPH) with O₂ followed by stopped-flow spectroscopy. The black solid line represents the first spectrum collected at 6.95 ms; the black dashed line represents the spectrum at 0.45 s and the black dotted line represents the spectrum at 45 s. Arrows point to the absorbance changes at different wavelengths and later used for analysis. Concentrations after mixing [FDP_F]= 30 μ M; [O₂]= 130 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

As before, the excess of O₂ allowed us to apply a pseudo-first order kinetic approach. As shown in Figure 42, the addition of O₂ led to a faster increase in both absorbances, 450 nm and 540 nm, during the first 0.3 s and a slower process for the remaining reaction time at 450 nm. In the remaining time up to 45 s, at 540 nm, no relevant changes were observed. The data was fitted with the sum of two exponentials for the absorbance at 450 nm, as described in equation 7, and was fitted with one exponential for the absorbance at 540 nm (Table 9).

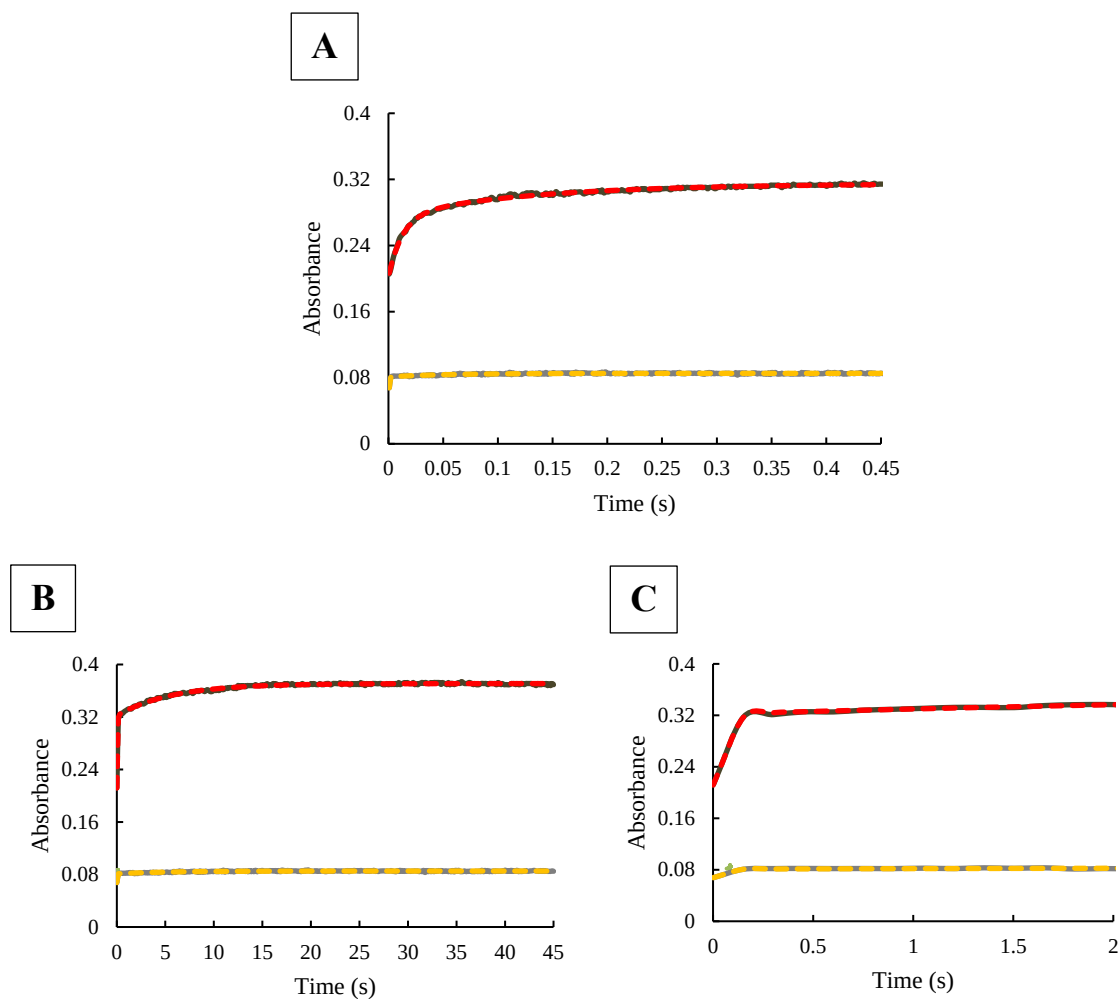


Figure 42: Typical traces and fits at 450 nm and 540 nm of FDP_F (reduced by NADPH) reacted with O₂ and followed by stopped-flow spectroscopy. Panel A- The black line represents the variation in the absorbance at 450 nm in the 0.45 s. The fit to the curve is represented by the red dashed line. The grey line represents the variation in the absorbance at 540 nm in the 0.45 s assay. The fit to the curve is represented by the orange dashed line. Panel B- The black line represents the variation in the absorbance at 450 nm in 45 s assay. The fit to the curve is represented by the red dashed line. The grey line represents the variation in the absorbance at 540 nm in 45 s assay. The fit to the curve is represented by the orange dashed line. Panel C- It is an amplification of Panel B up to 2 s. Concentrations after mixing [FDP_F]= 30 μ M; [O₂]= 130 μ M; T= 15°C, in 50mM Tris-HCl pH 7.5 buffer containing 18% glycerol.

Table 9: Calculated reaction rates of the FDP_F (reduced by NADPH) reacted with O₂ and followed at 450 nm and 540 nm by stopped-flow spectroscopy. The rates were calculated based on the variations of the absorbance at 450 nm over time. *Concentration after mixing.

Wavelength (nm)	[O ₂] (μ M)*	k ₁ (s ⁻¹)	k ₂ (s ⁻¹)
450	130	78.0 $\pm$ 0.5	6.7 $\pm$ 0.6
540	130	27.5 $\pm$ 1.8	-----

5 Discussion

Flavodiiron proteins have been identified as participants in the response mechanism to nitrosative and oxidative stresses in strict or facultative anaerobic microorganisms. Flavodiiron proteins present distinctive reactivities towards their different substrates such as O₂, NO and, as more recently discovered, H₂O₂. Some present higher turnover rates when oxygen is used as substrate while in others the turnover rates are comparable for both O₂ and NO. In fact, the only FDP that is described as having NO as its main substrate is the *Escherichia coli* flavorubredoxin [19] with a turnover rate about 10 times higher for this substrate when compared to O₂. [19] The fact that most of the studied FDPs are more reactive towards O₂ than NO is probably one of the reasons why the reaction mechanism of these enzymes for the NO reduction is still elusive. Over the past 15 years, several authors have used spectroscopic and theoretical approaches to propose different mechanisms for the NO reduction reaction in FDPs. [55-58]

During this thesis the main goal was to contribute to the characterization of the reaction mechanism of the NO reductase from *E. coli* and to uncover the reaction mechanisms of these proteins when oxygen is used as main substrate. Therefore, both the FDP from *E. coli* and the class F FDP from *Clostridioides difficile*, an O₂ reductase, were used during this study.

The biochemical characterization of Class B FIRD, its construct (FDP core) and Class F FDP (FDP_F) indicated that the general proprieties of these proteins, namely, iron and flavin content were in line with those previously reported by Vicente [59] and by Folgosa [22] for the *E. coli* FIRD and *C. difficile* FDP_F, respectively. During the reduction assays, when the enzymes were incubated with stoichiometric amounts of NADH, the UV-Visible spectra revealed that the amount of reductant used was lower than expected which is coherent with the lower amounts of iron and flavin per protein monomer that were determined.

After determining the proper reduction conditions for all the enzymes under study, obtaining the fully reduced and active form of the enzyme, without excess of electron donor was accomplished. Although the diiron center has a broad absorption maximum below 400 nm, most of the times it is not observed in the UV-Vis spectrum due to its low molecular absorptivity. [22] Therefore, the next step involved the study of the FDP-NO binding by EPR, which was achieved by incubating the reduced enzymes with different amounts of NO.

From the direct observation of the EPR spectrum for the FDP core, no signal corresponding to the diiron site in the mixed valence state (Fe^{II}Fe^{III}) was detected. In fact, besides the FMN radical, the only signal observed has g values of g=2.04 and 2.02, similar to what was observed previously by Kurtz and co-workers [60]. The fact that this signal is always present after the incubation with

2 eq. of NO points to the presence of a dead-end product from the reaction with the substrate, corresponding to an iron-dinitrosyl species.

In contrast, in the samples incubated with lower amounts of NO the EPR spectra of both F1Rd and FDP_F proteins show features attributable to the diiron site ($\sim g=1.96, 1.81$ and 1.78) in the mixed-valence state ($\text{Fe}^{\text{II}}\text{Fe}^{\text{III}}$). In fact, in the case of F1Rd, this signal disappears after incubation with 4 eq. of NO, indicating that the diiron site and also the other co-factors are fully oxidised, which is in accordance with the complete conversion of NO to its final product N_2O . In addition to these signals, also the one observed for the FDP core with $g=2.04$ was observed. However, no other signal indicative of the NO bound to the catalytic center or reaction intermediate was observed. This was probably due to the high efficiency of this enzyme in reducing NO.

On the other hand, in the case of FDP_F, the characteristic signal for the diiron center in the mixed valence state remains stable over the incubation with different additions of NO. This can be explained by the slower reaction of FDP_F with NO, which may also be responsible for the observation of other species such as the one with g values of $g=2.22$ and 1.73 that were identified in the sample incubated with 3 eq. of NO, that may correspond to an FDP-NO bound species, possibly an iron-mononitrosyl complex.[61]

Enzymes that catalyse redox reactions during the catalytic cycle undergo significant absorbance changes. In general, some transient intermediates are present, and the identification of these intermediates provides experimental information about the nature of the reacting species in catalysis but may form and decay too fast to be measured in standard spectrophotometers. Using a stopped-flow spectrophotometer it is possible to measure absorbance changes in the millisecond time scale.

The ability of reduced F1Rd to react with NO was investigated as previously described.[62] Since the stopped-flow assay with excess NO did not allow a complete oxidation of FMN and rubredoxin center, using an assay with a reduced amount of NO (ca. 10x dilution), it was possible to follow the behaviour of the FMN and rubredoxin domains. During catalysis with a lower NO concentration, the oxidation of the FMN co-factor and rubredoxin center demonstrates a rapid and exponential initial phase at about ~ 4 s, followed by a slower phase until reaching a plateau that persists for the remaining 45 s of the assay. This is demonstrated by the data shown in Figure 16 in Section 4.1.4, where the change in absorbance reflects 78 % and 42 % oxidation of FMN and rubredoxin, respectively, within the first 0.45 s. However, the fact that the calculated reaction rates were similar at both 450 and 540 nm suggests that both redox centers oxidize synchronously.

To determine if F1Rd follows a similar mechanism for the reduction of NO and O₂, the reaction with oxygen was also studied. During catalysis with O₂ as substrate, the oxidation of the FMN co-factor and rubredoxin center demonstrates a faster and exponential initial phase at about ~5 s, followed by a slower phase until reaching a plateau that persists for the remaining 45 s of the assay, Figure 25 in Section 4.2.4. Contrary to the reaction with NO, it was possible in this case, to distinguish a faster process with a $k'_1=121.4\text{ s}^{-1}$, Table 10, monitored at 450 nm, indicating that the FMN is the first co-factor to become oxidised after the diiron center, and only after that do the electrons flow from the rubredoxin center. This result demonstrates the relevance of the FMN domain to provide electrons to the reaction, which is of major relevance in the reduction of O₂ to H₂O, as it requires four-electrons that cannot be provided exclusively by a reduced catalytic diiron center.

Although it is not possible to make a direct comparison of the reaction rates obtained for F1Rd with NO and O₂ substrates, as these were performed with different concentrations of the two substrates, the fact that, in the reaction with NO, the first spectrum after mixing already shows a high amount of oxidised FMN, Figure 16 in Section 4.1.4, allowed us to speculate that the faster process of the reaction could not be observed, as it was for O₂, Figure 18 in Section 4.1.5, and, therefore, its reaction rate should be higher than the one observed for O₂.

The stopped-flow experiments with the FDP core reacting with an excess of NO and O₂ showed a similar behaviour to the F1Rd, having a faster initial phase and then a slower one. The results demonstrated an almost total oxidation of 91 % FMN with NO within 0.45 s. In fact, and as explained for F1Rd, this impairs a proper determination of the faster rate constants of this reaction, resulting in either undetectable processes or in highly underestimated rate constants. On the contrary, and as observed on the spectra of the reaction with O₂, Figure 27 in Chapter 4.2.5, it is possible to follow the spectral changes along the reoxidation. The faster process with a $k'_1=97.2\text{ s}^{-1}$, Table 10, is in the same range as the one determined for F1Rd, indicating that the absence of the rubredoxin domain does not interfere in the catalytic efficiency of the enzyme in a single turnover condition. From these experiments, it is also possible to say that the FDP core maintains NO as its preferential substrate, with a catalytic efficiency comparable to the F1Rd.

For this stopped-flow assay, both for the reaction with NO and O₂ reaction intermediates compounds were not detected for *E. coli* FDP.

Table 10: Calculated reaction rates of the FIRd and FDP core with O₂ followed at 450 nm by stopped flow spectroscopy. The rates were calculated based on the variations of the absorbance over time at 450 nm.

Protein	k_1 (s ⁻¹)	k_2 (s ⁻¹)
FIRd	121.4 ± 26.9	3.2 ± 2.8
FDP core	97.2 ± 1.9	9.6 ± 1.6

Class F FDP was also studied in terms of its NO reductase activity, not only to verify if FDP_F followed similar mechanisms for its main substrate, oxygen, but also to identify possible reaction intermediates as this enzyme is a homologue of FIRd. The ability of reduced FDP_F to react with different substrates was investigated and, as previously observed [62], being an oxygen reductase, it would be expected the rate constants obtained for the reaction with O₂ to be significantly higher than the ones observed for NO. With this enzyme, the reaction with O₂ resulted in a fast reoxidation of the FMN moiety immediately after mixing, impairing the determination of the faster rate constant. On the other hand, the reaction with NO not only allowed the monitoring of the complete reaction and, consequently, the determination of all the rate constants, but also demonstrated that the rubredoxin center did not oxidize during reaction when this substrate was used. This fact impairs a correct determination of the sequence of reoxidation of the co-factors and metal centers in this protein.

Another important observation from the reaction with both substrates is the apparent influence of the electron donor (NADH or NADPH) utilized. Although Folgosa [22] and co-workers have previously reported that NADH was the preferential substrate in steady-state kinetic assays, in single turnover experiments as the ones performed in this work, the use of NADPH as reducing agent promotes faster reaction rates when compared with when NADH is used, regardless of the substrate (O₂ or NO). This led us to infer that the NAD⁺ charge transfer complex may interfere directly with the reaction kinetics in these experimental conditions.

Overall, it should be noted that all proteins under study showed a similar behavior, a faster process corresponding to the partial (or almost complete in the cases of FIRd and FDP reacted with NO) oxidation of FMN, indicating that the electrons utilized during catalysis by the diiron catalytic site are quickly replaced, and then a slower process, corresponding to the remaining reoxidation of the FMN moiety and/or rubredoxin domain when it is present. This slower kinetic phase may also be related either with the contribution of a minor subpopulation of the enzyme with lower levels of iron or FMN incorporation [63], or with the accumulation of small amounts of anionic

FMN_{SQ} [57], although in all the proteins under study, no FMN_{SQ} intermediate was observed during catalysis in single turnover conditions.

Our cumulative results on *E. coli* FDP provide a paradigm for FDP catalysis. Based on the results presented in this thesis it is possible to understand the reduction of NO, O₂ and the binding of NO to these proteins, especially to the FDP_F, where the EPR signals are observable.

By combining the results described in this thesis, namely the absence of EPR signals upon the anaerobic incubation with small amounts of NO, it is possible to infer the formation of a dinitrosyl [(FeNO)⁷]₂ intermediate, perhaps via the formation of a mononitrosyl [(Fe^{II}NO)⁷] intermediate as suggested by Kurtz and co-workers [57] although no spectroscopic evidence for this species was obtained in this work. Therefore, these data agree with the proposed mechanism by Kurtz and co-workers regarding the catalytic cycle of these enzymes for the reduction of NO, summarized in Figure 43.

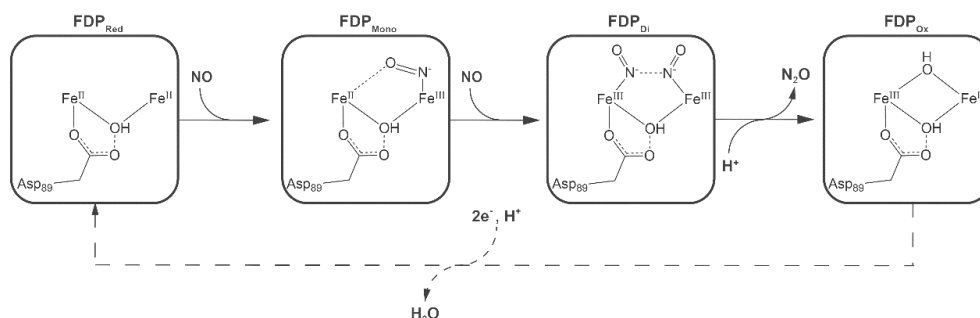


Figure 43: Catalytic mechanism of diiron center with NO substrate, proposed by Kurtz and co-workers. FDP_{red} reaction sequential binding of 2 NOs with formation of diferrous mononitrosyl and diferrous dinitrosyl intermediates, leading to the formation of FIRD_{ox} and N₂O.

However, the data obtained in this thesis may point to a direction contrary to Kurtz's proposal regarding the influence of the FMN moiety. Kurtz proposed that FMN has a role in the transfer of electrons to the diferric site only after the release of the product [57], not affecting the NO reduction reaction. In contrast, our results suggest that FMN plays a crucial role during the reaction since the incubation of substoichiometric amounts (under 2 eq.) of NO to the reduced protein leads to the formation of the FMN_{SQ}, observed in the EPR experiments. This may indicate that either electron transfer occurs to the diiron center during catalysis or there is cooperativity in the NO binding, allowing the product formation with low substrate amounts. The high reaction rates for the FMN reoxidation determined in the stopped-flow experiments, and in the

experiments where O₂ was used as substrate, also point to a fundamental role of this moiety during catalysis. Further studies should be carried out to fully understand the role of FMN in the catalytic pathway of FDPs.

6 Conclusion

The work summarized herein describes several experimental methodologies that altogether contributed to the functional and mechanistic characterization of the FDP from *E. coli*.

By combining biochemical and spectroscopic methodologies to the study of *E. coli* FDP, it was possible to conclude that, although it has an activity for two substrates (oxygen and nitric oxide), the preferential substrate, under single turnover conditions, was determined to be NO, which is reduced to N₂O, in agreement with its reported physiological role. Based on the remarkable reactivity to NO, we propose that during the catalysis, the binding of NO occurs sequentially and with the contribution of the different centers, more specifically with the direct participation of the FMN. The experimental data points to the possibility that FMN participates in electron transfer to the diiron center during catalysis, which takes place via the formation of a dinitrosyl [(FeNO)⁷]₂ intermediate.

Thus, it is concluded that the Class B FDP from *E. coli* can indeed play a crucial role in the detoxification of NO, allowing microorganisms to survive in the human intestine and the environment, thus promoting their pathogenicity.

With this work, we directly contribute to the advance in the functional characterization of FDPs with the preferred substrate, NO. However, in the future it would be essential to perform further kinetic assays using not only different NO concentrations but also at lower temperatures to determine the real kinetic rates. As a complement to the study of the catalytic center, the use of other spectroscopies such as Mössbauer spectroscopy and density functional theory would be interesting to better understand the binding of NO to the catalytic center and thus provide more information about possible intermediates.

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8 Appendices

Appendix 8.1- Transformation protocol

The transformation protocol used throughout this work was slightly adapted from the NZYtech® protocol.

The BL21(DE3) chemically competent *Escherichia coli* cells used were stored at -80 °C.

1. Thaw competent cells on ice. Gently mix cells. Do not mix cells by pipetting.
2. In 2 separate microcentrifuge tubes, add 40 µL of BL21(DE3) competent cells, one is used as positive control and the other for the protein over expression.
3. Add 4 µL of the plasmid DNA to the protein over expression microcentrifuge tubes.
4. Incubate cells on ice for 30 minutes.
5. Heat-shock cells for 40 seconds in a 42 °C water bath; do not shake.
6. Place on ice for 2 minutes.
7. Add 900 µL room temperature LB medium.
8. Shake at 160 rpm (37 °C) for 90 mins at 37 °C.
9. On 3 different LB agar plates containing kanamycin, 1- spread 100 µL of cells transformed with competent cells with DNA plasmid; 2 - spread 200 µL of cells transformed with competent cells with DNA plasmid; 3 - spread 200 µL of cells transformed with competent cells without DNA plasmid.
11. Incubate overnight at 37 °C in oven.

Appendix 8.2- Medium composition.

Appendix 8.2.1- Liquid LB medium composition.

Reagent	Mass/ Volume
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
H ₂ O Milli-Q	Up to 1000 mL

Appendix 8.2.2- LB agar medium composition.

Reagent	Mass/ Volume
Tryptone	3 g
Yeast Extract	1.5 g
NaCl	3 g
Agar	6
H ₂ O Milli-Q	Up to 300 mL

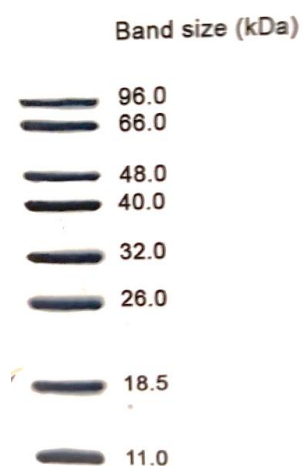
Appendix 8.2.3- M9 Salts composition.

Reagent	Mass/ Volume
Na ₂ HPO ₄ .12H ₂ O	170 g
KH ₂ PO ₄	30 g
NH ₄ Cl	10 g
NaCl	5 g
H ₂ O Milli-Q	Up to 2000 mL

Appendix 8.1.4- M9 Medium composition.

Reagents	Volume
M9 salts (x5)	400 mL
MgSO ₄ (1M)	4 mL
CaCl ₂ (5M)	0.04 mL
Glucose (1M)	40 mL
FeCl ₂ (0.1M)	2 mL + 2 mL together with IPTG)
H ₂ O Milli-Q	Up to 2000 mL
IPTG	0.2 mL

Appendix 8.3- Molecular mass markers of the NZYTech protein marker II (molecular mass from 11 to 96 kDa).



Appendix 8.4- SDS-PAGE solutions

Appendix 8.4.1- SDS-PAGE composition (Resolving gel).

Resolving gel	
Reagent	Volume (μL) 15%
Solution II	2500
Acrylamide	5000
dH ₂ O	2050
10% APS	40
TEMED	10

Appendix 8.4.2- SDS-PAGE composition (Stacking gel).

Stacking gel	
Reagent	Volume (μL)
Solution II	1250
Acrylamide	650
dH ₂ O	3050
10% APS	40
TEMED	10

Appendix 8.4.3- Coomassie Brilliant Blue protein staining.

Reagent	Mass/ Volume
Coomassie Blue R-250	1 g
Glacial acetic acid	15 mL
Methanol	90 mL
dH ₂ O	Up to 200 mL

Appendix 8.4.4- Destaining solution

Reagent	Volume
Glacial acetic acid	75 mL
Methanol	450 mL
dH ₂ O	Up to 1000 mL