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MINIREVIEW

The dual function of flavodiiron proteins: oxygen and/or nitric oxide reductases

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Abstract Flavodiiron proteins have emerged in the last two decades as a newly discovered family of oxygen and/or nitric oxide reductases widespread in the three life domains, and present in both aerobic and anaerobic organisms. Herein we present the main features of these fascinating enzymes, with a particular emphasis on the metal sites, as more appropriate for this special issue in memory of the exceptional bioinorganic scientist R. J. P. Williams who pioneered the notion of (metal) element availability-driven evolution. We also compare the flavodiiron proteins with the other oxygen and nitric oxide reductases known until now, highlighting how throughout evolution Nature arrived at different solutions for similar functions, in some cases adding extra features, such as energy conservation. These enzymes are an example of the (bioinorganic) unpredictable diversity of the living world.

Keywords Flavodiiron · Diiron · Oxygen · Nitric oxide · Reactive oxygen species

Abbreviations

AOX	Alternative oxidase
FDP	Flavodiiron protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

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Introduction

Transition from an anaerobic to an aerobic world

At the age of 20, inspired by Darwin's "The Voyage of the Beagle", R. J. P. Williams hypothesized that Life's evolution depended on optimising the use of all available elements. The idea that "the elements to be found in organisms were used to the best advantage of living systems" implied that these elements were intensely selected and used [1]. Life originated on Earth ca 3.5 billion years ago in an anoxic and reducing environment that sustained different types of anaerobic metabolisms. Molecular oxygen, if present, would be vestigial and confined to restricted niches. This situation changed dramatically upon appearance of oxygenic photosynthesis performed by cyanobacteria, leading several million years after to what has been called the Great Oxidation Event, about 2.5 billion years ago. Oxygen in the atmosphere eventually stabilized at around 21 %, i.e., a quite oxidising environment was established, leading to major changes in the cellular metabolism. Existing life forms adopted different strategies to cope with this new challenge: (1) avoiding it by inhabiting anaerobic niches, but "evolving" (possibly using already existing cellular components) ways to protect themselves while keeping the intracellular reducing medium, or (2) profiting from it, both for biosynthetic processes and for aerobic respiration. In fact, the highly positive oxygen reduction potential (+0.82 V at standard atmospheric pressure and temperature) allowed extraction of maximum energy from cellular food stuff (e.g., glucose), which favoured the evolution of the complex multicellular life forms now present on Earth. However, oxygen also poses a threat to either anaerobic or aerobic living forms (the 'dark side' of oxygen): although

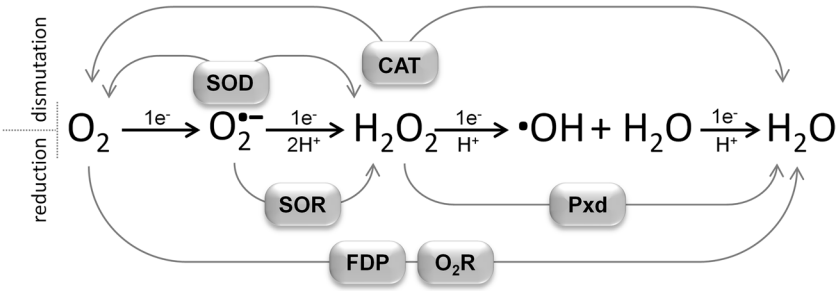


Fig. 1 Enzymatic systems for detoxification of oxygen and reactive oxygen species. *SOD* superoxide dismutase, *CAT* catalase, *SOR* superoxide reductase, *Pxd* peroxidases, *FDP* flavodiiron proteins, O_2R respiratory oxygen reductases

the oxygen reactivity is limited by its spin triplet ground state, it may still react fairly rapidly with radical organic compounds, such as flavins or quinones, or with metals, such as the widespread iron centres. Moreover, upon successive one electron reduction steps, oxygen forms highly reactive species (ROS), the superoxide anion, hydrogen peroxide and the hydroxyl radical, that may further and rapidly react with multiple cellular components (again, metal centres, thiols, lipids and nucleotides). As a result, all cellular forms analysed to date contain enzymatic systems to sense, use, and eliminate molecular oxygen and the derived reactive species (Fig. 1). Molecular oxygen can be fully reduced to water by respiratory membrane-bound oxygen reductases (haem-copper reductases, alternative oxidase (AOX) and cytochrome *bd*) and O_2-reducing non-haem diiron enzymes, the flavodiiron proteins (FDPs), the subject of this review.

The biochemistry of NO

Nitric oxide plays key roles in the living world [2]. Abiotic NO production results mostly from nitrite acidification, for example in the mammalian gut or during hypoxia [3]. It may also be enzymatically generated, mainly by nitric oxide synthases [4] from multi-cellular organisms and some bacteria, or as an intermediate in microbial nitrogen metabolic pathways: denitrification and anaerobic ammonium oxidation (Anammox) [5, 6]. At low concentrations (in the nanomolar range) it plays important roles as a signalling molecule regulating many physiological processes. However, at higher concentrations it is quite harmful, and under aerobic conditions it rapidly reacts both with oxygen and ROS producing even more reactive molecules; in fact, responses to oxidative and nitrosative stresses are intimately related. Altogether, NO and reactive nitrogen species (RNS) target key cellular components such as amino acids, nucleic acids, lipids, thiols and metal centres, thereby inhibiting a plethora of metabolic pathways. A remarkable role of NO in multicellular eukaryotes concerns its production by the immune system to combat invading pathogens [7]. In this endless strife, it is no surprise that many microorganisms may cope with such immune system weapons by having diverse ways to sense and detoxify NO, an example being the flavodiiron proteins.

Flavodiiron proteins: a short historical account

Over the past two decades, the flavodiiron proteins have increasingly attracted the interest of the bioinorganic chemistry community. Highlights of the short history of this enzyme family are depicted in Fig. 2. The first reported flavodiiron protein, and for which a function was promptly proposed, was isolated in 1993 from the then considered strict anaerobic bacterium *Desulfovibrio* (*D.*) *gigas*. *D. gigas* FDP was initially named rubredoxin:oxygen oxidoreductase (ROO), as it coupled the oxidation of rubredoxin (reduced by an NADH oxidoreductase) to oxygen reduction to water [8]. Based on an amino acid sequence analysis, Wasserfallen et al. identified a new family of flavo(metallo)enzymes, then named A-type flavoproteins (FprA), and did a basic characterization of *Escherichia coli* and *Synechocystis* FDPs, proposing at the time a classification based on the different cofactors (domains) [9]. A landmark in FDP history was the determination of the crystallographic structure of *D. gigas* FDP in 2000 (detailed below), which revealed for the first time the existence of the β-lactamase-like fold harbouring a non-haem diiron centre [10]. Whereas most focus was given to the oxygen detoxification role, a striking observation concerned the proposal by Gardner et al. that *E. coli* FDP is an NO reductase [11], soon after confirmed by amperometric measurements with the isolated enzyme [12]. In 2003, Kurtz and co-workers reported the first example of a bi-functional FDP (from *Moorella thermoacetica*) endowed with both NO and O_2 reductase activity, and coined the term "Flavodiiron protein" for this enzyme family [13]. In the meantime, a significant line of research in the FDP field appeared, concerning the involvement of cyanobacterial FDPs in photoprotection of oxygenic photosynthesis (e.g., [14, 15]). Over the last 10 years, other bifunctional FDPs were characterised [16, 17] as well as FDPs more selective either for NO (*E. coli* and *Salmonella enterica* FDPs) or for oxygen (e.g., eukaryotic FDPs from anaerobic protozoa or from methanogens) (Table 1). Many structural and biophysical data have since

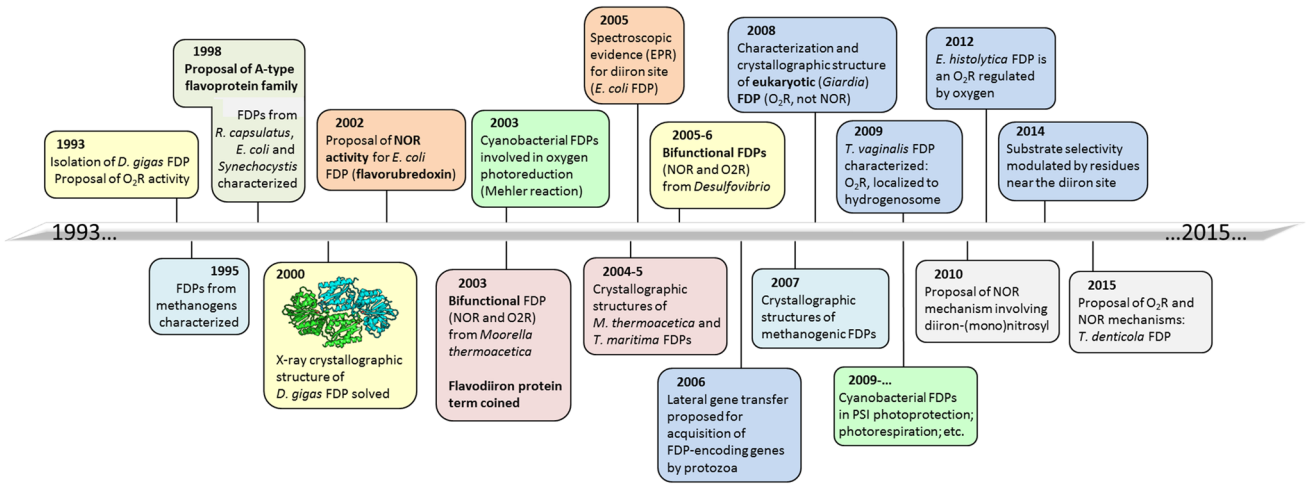


Fig. 2 Timeline depicting the main scientific landmarks concerning the history of the flavodiiron protein family. Adapted from [103]

accumulated, which have provided insights into the reaction mechanisms both for O_2 and NO reduction, as well as on structural determinants for substrate preference within this protein family.

Presently, FDPs are recognised as a large family of enzymes, widespread in all life Domains (Bacteria, Archaea and Eukarya) (Table 1), with a key function in oxygen and/or nitric oxide detoxification, as herein illustrated.

The FDP family and their modular nature

The FDP minimal structural unit is composed by two domains: the N-terminus is homologous to metallo-β-lactamases and the C-terminus is similar to small-flavodoxins (details of the structure are described below). These two domains constitute the common denominator of this enzyme family, and gave rise to the general term Flavodiiron Proteins (FDPs) (Fig. 3).

This minimal common denominator is present in the Class A FDPs, the most widespread among prokaryotes [18, 19]. Yet, more complex and interesting modular structures have been studied or encountered in the genomes, with extra domains at the C-terminal part of the protein (Fig. 3a): rubredoxins (Class B FDPs, so-called flavorubredoxins, so far only found in the Proteobacteria phylum, particularly in Enterobacteriales), flavin reductases (Class C FDPs, in all cyanobacteria whose genomes have been sequenced, as well as in several photosynthetic eukaryotes), and the putative Class D and E enzymes (not yet isolated, their domain composition being derived from their gene sequence), with both extra rubredoxin and flavin reductase/NAD(P)H:rubredoxin oxidoreductase-like domains in some protozoa and clostridiales [20–22].

Another interesting feature of FDPs concerns the electron transfer chains supplying electrons to FDPs to act as O_2/NO reductases (Fig. 3b). NAD(P)H appears to be the common primary electron source, the sole exception being the F_{420} cofactor in methanogenic Archaea, which directly reduces the *Methanobrevibacter arboriphilus* and *Methanothermobacter thermoautotrophicum* FDPs [23, 24]. A common feature of several FDPs is the ability to oxidise rubredoxins, small electron transfer proteins with a [$FeCys_4$] centre, which are reduced by flavin-containing NAD(P)H oxidoreductases. In the protozoan pathogen *Entamoeba histolytica*, amoebic NAD(P)H-oxidising flavoprotein and ferredoxins have been shown to be electron donors to EhFdp1, this parasite's Class A FDP [25].

Class B FDPs have the rubredoxin fused to the C-terminus as an extra domain, bypassing the need for an external rubredoxin partner. Similarly, Class C FDPs have an extra C-terminal flavin reductase-like domain that directly accepts electrons from NAD(P)H, thus condensing the electron transfer chain into a single polypeptide [26]. Although still uncharacterised, the modular nature of Classes D and E FDPs also seems to eliminate the need for extrinsic redox protein partners (Fig. 3b). It should be stressed that in many instances the physiological electron donor to the FDPs remains unknown, particularly in organisms whose genomes lack genes encoding rubredoxins.

The structure of flavodiiron proteins

The flavodiiron proteins are isolated as homodimers or homotetramers (dimer of dimers), and their crystallographic structures confirm this quaternary arrangement (Table 1). An exception occurs in cyanobacteria, where FDP heterodimers assembled from homologous monomers have been proposed [27]. In Class A FDPs each 40–48 kDa monomer is constituted by two domains: the N-terminal

Table 1 Information on FDPs so far studied

Class	Organism	Group	Quaternary structure	Activities		X-ray crystal structure			Redox potential			References
				O_2R	NOR	PDB	Proposed redox state	Resolution (Å)	E^{FMN} (mV)	$E^{Fe–Fe}$ (mV)	E^{Rd} (mV)	
A	*D. gigas*	Anaerobic	Dimer	Yes	Yes	1E5D	Oxidised	2.50	0/−130	n.d	(a)	[8, 16, 107]
	D. vulgaris	Anaerobic	Dimer	Yes	Yes	–	–	–	–	–	(a)	[17]
	M. thermoacetica	Anaerobic	Dimer	Yes	Yes	1YCF	Oxidised	3.00	−117/−220	n.d	(a)	[13, 17]
						1YCG	Reduced	2.80				
						1YCH	No-reacted	2.80				
	C. acetobutylicum FDP_2	Anaerobic	Tetramer	Yes	Yes	–	–	–	–	–	(a)	[62, 63, 108]
	G. intestinalis	Anaerobic	Tetramer	Yes	No	2Q9U	Oxidised	1.90	−66/−83	+2/+163	(a)	[42, 54]
	T. vaginalis	Anaerobic	Dimer	Yes	No	–	–	–	+25/+25	+50/+190	(a)	[40]
	E. histolytica	Anaerobic	Dimer	Yes	No	–	–	–	−55/−140	+170/+132	(a)	[43]
	C. acetobutylicum FDP_1	Anaerobic	Dimer	Yes	No	–	–	–	–	–	(a)	[62, 63, 108]
	M. thermoauto-trophicum	Anaerobic	Tetramer	Yes	No	2OHH	Oxidised	1.70	–	–	(a)	[24, 109]
						2OHI	Reduced	2.30				
						2OHJ	Inactive oxidised	2.26				
	M. arboriphilus	Anaerobic	n.d.	Yes	No	–	–	–	–	–	(a)	[23]
						1VME	Oxidised	1.80	–	–	(a)	[53, 59]
	T. maritima	Anaerobic	Dimer	Yes	No	4DIK	Mut H90A	1.75	–	–	(a)	
						4DIL	Mut H90N	2.00	–	–	(a)	
	Rhodobacter capsulatus	Anaerobic	Dimer	Yes	No	–	–	–	+20 mV (pH 8.0)	–	(a)	[109, 110]
B	*E. coli*	Facultative anaerobic	Tetramer	No	Yes	4D02	Oxidised	1.75	−40/−130	−20/−90	−123	[9, 12, 41]
C	*Synechocystis* Flv3	Aerobic	Dimer	Yes	No	–	–	–	–	–	(a)	[9, 26]

O_2R oxygen reductase, *NOR* nitric oxide reductase, *n.d.* not determined, *(a)* absent

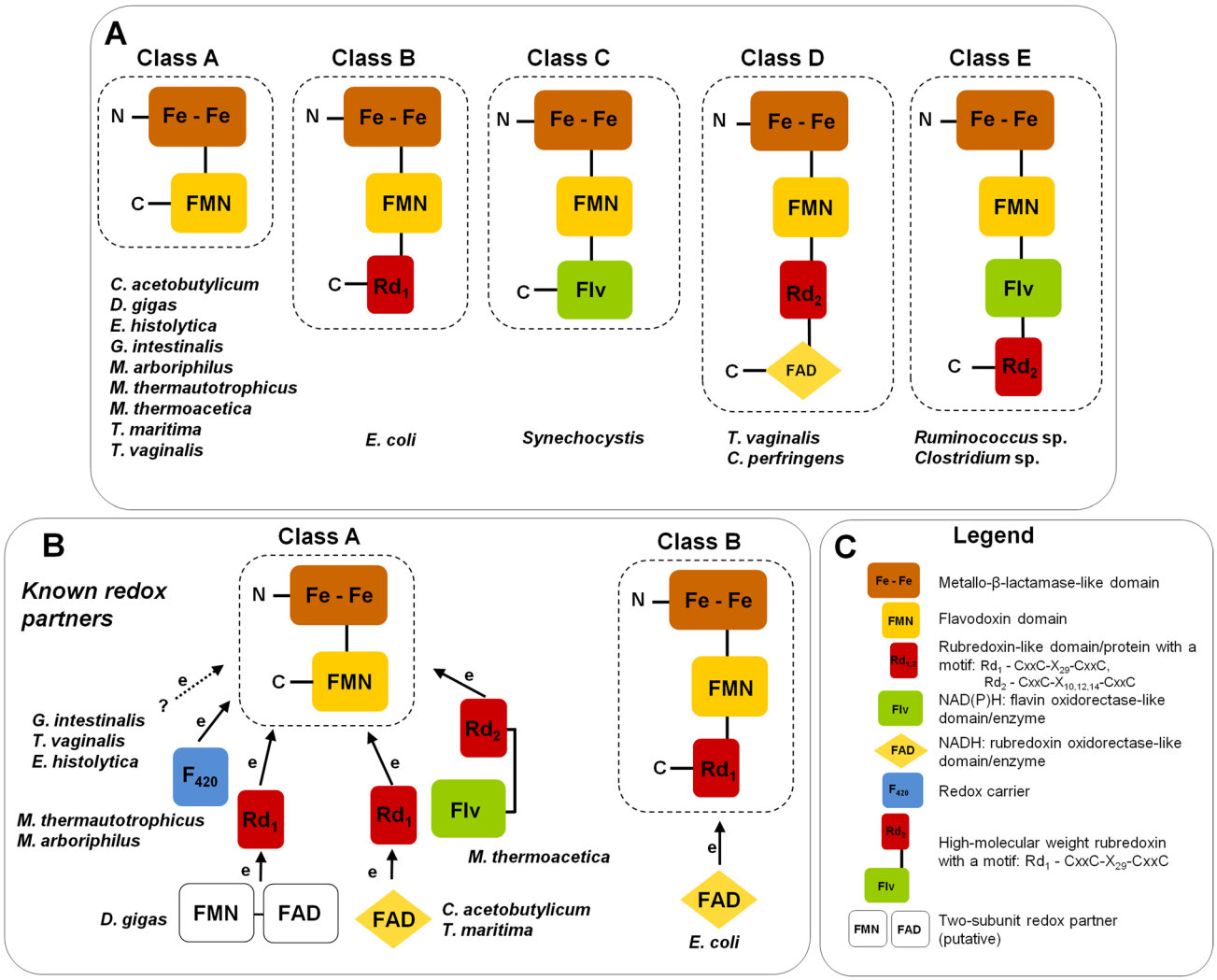


Fig. 3 The flavodiiron protein family. **a** FDP classification according to the known or proposed domain structure (deduced from their gene-translated amino acid sequences). **b** Scheme of the electron transfer chains comprising the known redox partners of FDPs. **c** Legend for the different domains. Adapted from [103]

domain shows an αββα topology characteristic of metallo-β-lactamase-like folds, harbouring a diiron centre; and the C-terminal domain with an αβα topology similar to short-chain flavodoxins that binds non-covalently a flavin mononucleotide (FMN) (Fig. 4a, b). The distance between the diiron centre and the FMN within the same monomer is ca. 40 Å, precluding direct electron transfer. However, the minimal functional unit in structurally characterised FDPs is a dimer arranged in a head-to-tail orientation, in which the diiron centre from one subunit is at ca. 6 Å from the FMN from the other monomer, thus ensuring an efficient electron transfer between the two centres (Fig. 4c).

Structures homologous to FDPs common denominator components, metallo β-lactamase-like and flavodoxin-like domains, have been found as individual molecules or together with domains from other composite enzymes. Metallo-β-lactamases may harbour various metals such as iron, zinc or manganese, in mononuclear or binuclear centres, with single or mixed metals, and catalyse redox or hydrolytic reactions [28]. Glyoxalases and persulfide dioxygenases are examples of such redox enzymes, while methyl parathion hydrolase, phosphorylcholine esterase, N-acyl homoserine lactone hydrolase, alkyl sulfatase, and the β-CASP family are examples of hydrolytic enzymes whose structures contain metallo-β-lactamase-like domains [29–34]. Despite the low amino acid sequence identities (within 7–24 %), there is a remarkable 3D superposition between those lactamases, the metallo-β-lactamase from *Stenotrophomonas maltophilia* and the *M. thermocetica* FDP metallo-β-lactamase-like domain (Cα rmsd's within 1.6–2.6 Å for 3.5 Å Cα distances cut-off). Similarly, flavodoxin-like domains were found in

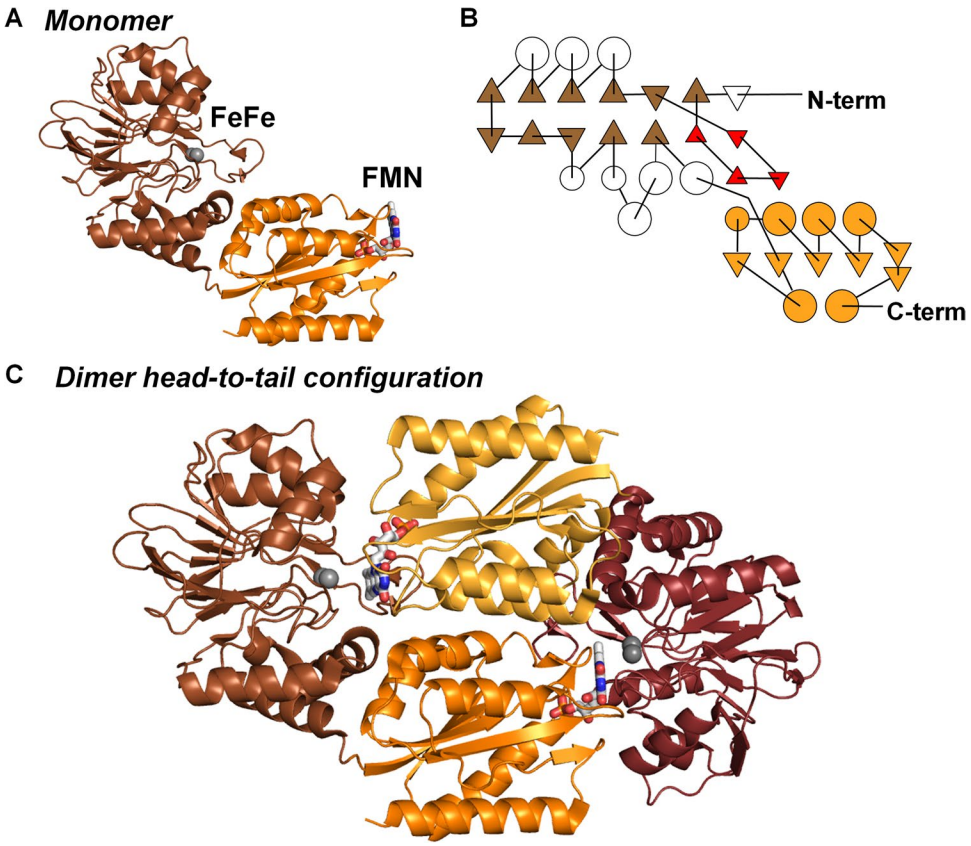


Fig. 4 Overall structure of flavodiiron proteins. **a** Crystallographic structure of FDP representative (*Moorella thermoacetica* FDP, PDB code 1YCF), with the N-terminal metallo β-lactamase like domain in *brown* and the C-terminal flavodoxin domain in *orange* represented in cartoon. **b** Topology diagram of FDP representative, with *circles* and *triangles* representing α-helices and β-chains, respectively. Fillings in *brown* and *yellow* highlight the conserved secondary structural elements in β-lactamase and flavodoxin domains, respectively, and the filling in *red* highlights the additional β-chains in β-lactamase like domains unique to FDPs. **c** FDP representative in its dimeric head-to-tail configuration, with N-terminal metallo β-lactamase like domains in *brown* and C-terminal flavodoxin domains in *orange* represented in cartoon. Iron atoms are represented as *gray spheres* and FMN as *bond sticks*. Figure prepared with Pymol [104, 105]. All remaining structural figures were prepared with this molecular graphic software

sulphite reductase, cytochrome P450 reductases and nitric oxide synthase [35–38]. Their 3D superposition (up to a 3.5 Å Cα distances cut-off) together with the short-chain flavodoxin from *Clostridium beijerinckii* and the flavodoxin-like domain from *M. thermocetica* FDP led to Cα rmsds of 0.91–2.21 Å (amino acid sequences identities of 13–32 %).

The diiron centre of flavodiiron proteins

The binuclear site of FDPs is located within a groove at the interface between the two inner β-sheets of the β-lactamase-like fold. It is surrounded by αβ loops and β-hairpins, being in the vicinity and confined by the C-terminal domain of the opposing monomer. Differently from other metallo-β-lactamase-like proteins, the metal site is covered by a unique two-stranded β-sheet that, together with the dimer mate, hinders the access of large substrates, such as β-lactams (Fig. 4b). Indeed, several β-lactams have been tested and failed to inhibit FDPs (Vicente and Teixeira, unpublished data).

The FDP crystal structures show that the amino acid metal ligands are almost strictly conserved. The diiron centre is coordinated by a μ-(hydr)oxo species, three carboxylate and four imidazole ligands (Fig. 5), from a highly conserved motif, H^{81}-x-E^{83}-x-D^{85}-H^{86}-x_{61}-H^{148}-X_{18}-D^{167}-x_{60}-H^{228} (*Moorella thermoacetica* FDP numbering, henceforth used unless stated otherwise). The iron atom closest to the FMN (proximal iron, Fe_P) is coordinated by His81, Glu83 and His148, while the distal atom is bound to Asp85, His86 and His228. Both irons are further bridged by the carboxylate group of Asp167 and by the μ-(hydr)oxo species (Fig. 5). In *D. gigas* FDP a water molecule substitutes the His84 imidazole, which adopts a unique chi1 value allowing the establishment of an additional hydrogen bond network involving Asp225, Asp49 and Gln78 (*D.*

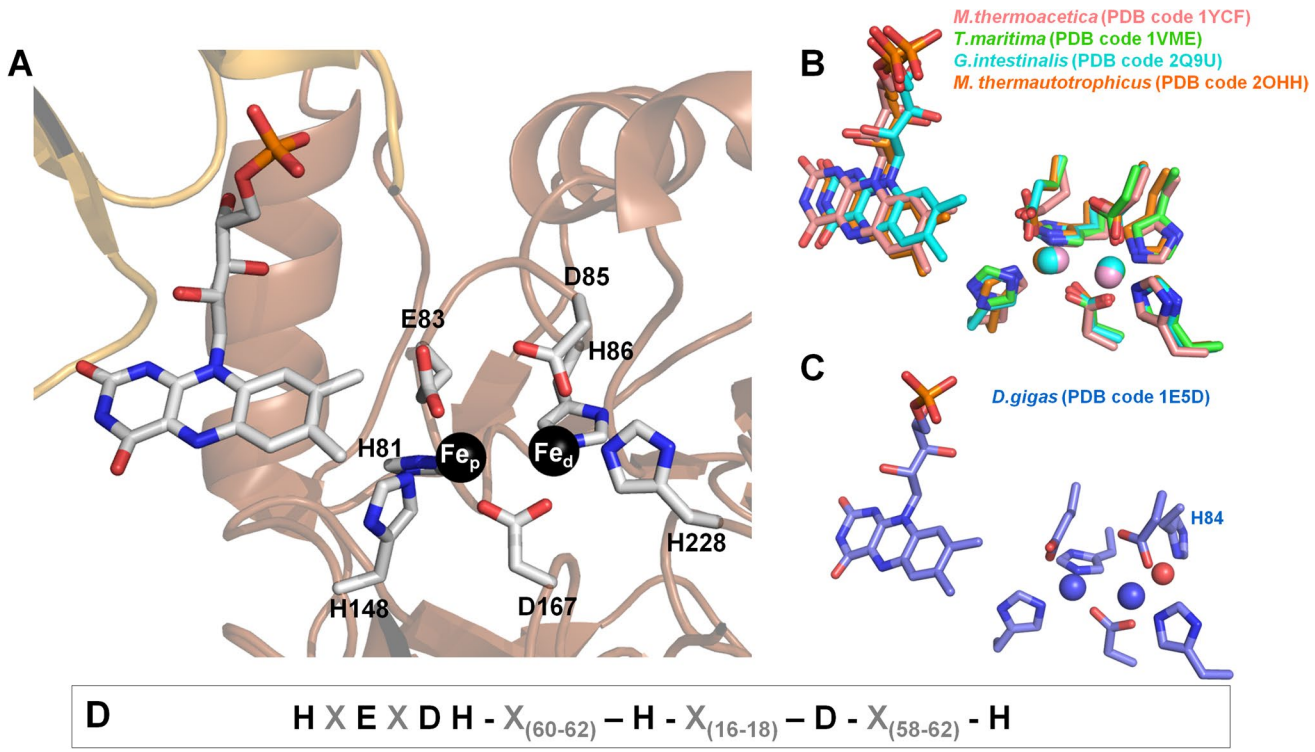


Fig. 5 Structure of diiron catalytic centre in flavodiiron proteins. **a** *Moorella thermoacetica* FDP (PDB code 1YCF) diiron centre, with iron atoms as *black spheres* labelled Fe_p and Fe_d, denoting their proximal and distal positions relative to FMN, respectively; atomic bonds of iron ligands and FMN as *sticks*. *Pink* and *orange* cartoons colours differentiate the two monomers. The μ-solvent moiety and the oxygen molecule which are bridging the two iron atoms are not represented. **b** Superposition of FDPs diiron sites represented by *sticks* (bonds) and *spheres* (atoms) with carbons and irons colour coded in *pink* for *Moorella thermoacetica* FDP, in *green* for *Thermotoga maritima* FDP, in *cyan* for *Giardia intestinalis* FDP, and in *orange* for *Methanothermobacter thermautotrophicum* FDP. **c** The *Desulfovibrio gigas* FDP diiron centre, where His84 is not an iron ligand but is replaced by a water molecule (*red sphere*), with colour codes as in previous panel except for carbons and iron, here in *light blue* and *blue*, respectively. **d** FDPs amino-acid residues motif for iron binding based on the consensus of available crystal structures. The structural superposition was obtained using Modeller [106]

gigas FDP numbering). In the available set of FDP structures, iron atoms are on average at 3.4 (3.2–3.7 range) Å or at 3.8 (3.4–4.0 range) Å apart from each other, for the oxidised or reduced forms, respectively. The two metal sites are penta-coordinated with distorted square pyramidal geometries, with the bases of the pyramids approximately parallel to each other. Most of the structures of oxidised FDPs show molecules modelled as nitrate, sulphate, water or dioxygen close to the base of the pyramids, within 2.1–3.6 Å distance to the closest iron position. These molecules are either approximately equidistant to both iron atoms or located closer to the distal iron, when they seem to mimic a distorted octahedral geometry. As already mentioned, the head-to-tail configuration allows an efficient electron transfer between the two redox centres, via the methyl group C8M of the FMN of one monomer that is in close proximity (ca. 3.5 Å) to the carboxylate from Glu83, a ligand of the proximal iron (Fig. 5a) [10, 39].

Most Class C enzymes may have a distinct iron coordination, as some ligands are not conserved, being eventually substituted by non-canonical ligands such as arginines and glutamines. They show a large variability in the combination of these amino acids in the positions for possible iron binding, which led to the classification of 15 different subtypes of Class C enzymes [19]. The only available structure of a Class C truncated enzyme that could contain the metal site [metallo-β-lactamase-like domain of a FDP from *Anabaena* sp. 7120, All0177 (PDB code 4FEK)] showed the site to be demetallated. This leaves as an open question whether these enzymes have indeed non-canonical ligands to the metal site or in fact contain only the flavin cofactor, which raises another question regarding their catalytic function.

As in other diiron proteins, the iron ions in the FDPs are antiferromagnetically coupled, as observed by Mössbauer spectroscopy [13]. This property makes the diiron centre EPR silent in the as-isolated ferric state. However, in the mixed-valence [Fe^{III}–Fe^{II}] form, they exhibit rhombic spectra with g values below 2.0 [40–44], characteristic of this type of centres [45]. This spectroscopic signature allowed

probing the redox properties of the diiron centres in several FDPs [40–44].

FDPs and other diiron proteins

There is a wealth of diiron proteins displaying the most diverse functions, such as monooxygenases, ribonucleotide reductases, oxygen transporters (haemerythrins) or reducers (alternative oxidases, see below), and ferroxidases (ferritins and haemferritins) [46–49]. Despite containing diiron centres of the histidine/carboxylate family like those of FDPs, all those referred proteins contain their metal site embedded in a four-helix bundle fold. It should be mentioned that other diiron containing proteins have been discovered with distinct folds, such as mammalian purple acid phosphatases (e.g., [50]) or amino acid β-hydroxylases involved in antibiotic biosynthesis (these hydroxylases include the metallo-lactamase-like fold, where the diiron centre resides, as in FDPs, e.g., [51]). Although the number of histidine and carboxylate ligands also varies between these different proteins, both FDPs and the mentioned four-helix-bundle diiron proteins have in common that they activate/react with oxygen, be it for its transport (haemerythrins), to oxidise ferrous iron (ferritin) or hydrocarbons (e.g., methane monooxygenase), or to generate tyrosyl radicals (ribonucleotide reductases). Only alternative oxidases have as their physiological function the direct reduction of oxygen to water. However, the general biophysical properties of the metal centre are similar, including the existence of the diiron centre in three redox states (diferric, mixed valence and diferrous), within the same range of reduction potentials (between ca. −100 to +250 mV). It remains to be established what determines the diverse specific activities in such structurally similar diiron centres, including those of FDPs, but the local protein environment certainly plays a pivotal role. In this respect, it is interesting to note that NO is known to bind to several diiron centres but, to our knowledge, no NO reductase activity was never reported for other diiron-containing proteins besides FDPs.

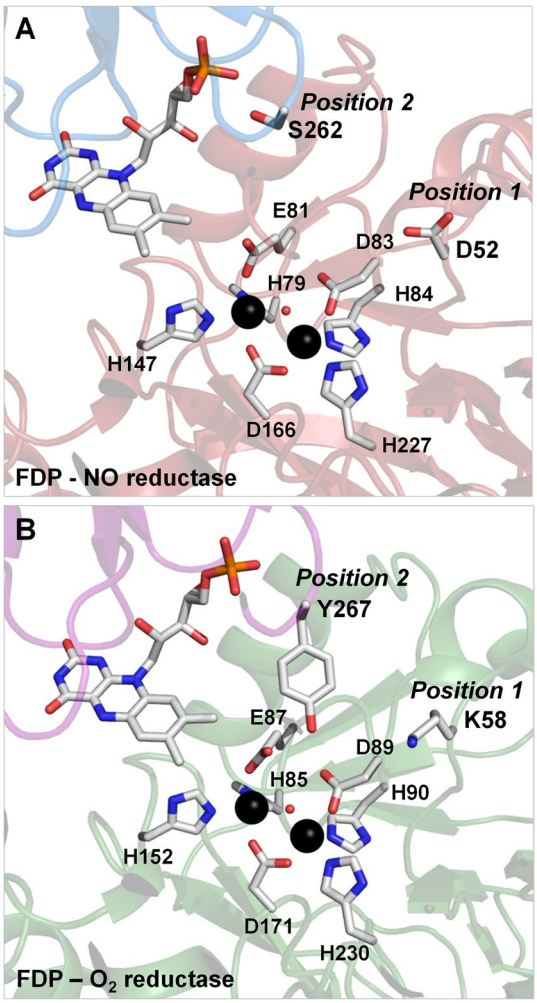
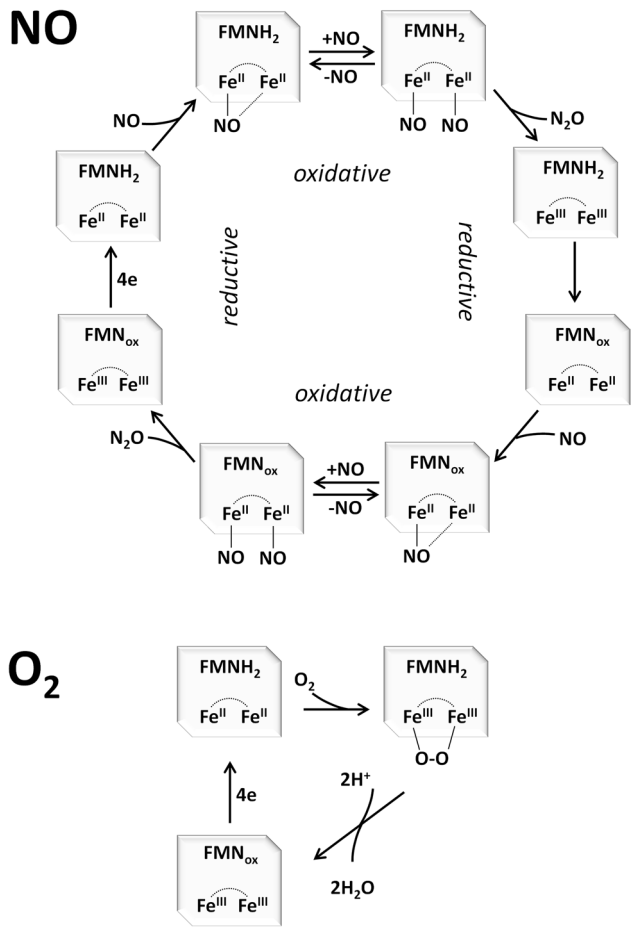


Fig. 6 Diiron sites structural surrounding in FDPs with different activity: NO reductase versus O_2 reductase. **a** Diiron centre region of the NO reducing *Escherichia coli* FDP (PDB code 4D02). **b** Diiron centre region of the O_2-reducing *Giardia intestinalis* FDP (PDB code 2Q9U). In both panels the head-to-tail oriented monomers are differentiated with distinct colours, bonds are represented as *sticks*. The μ(hydr)oxo-bridging oxygen moiety and iron atoms are represented as *red* and *black spheres*, respectively. Positions 1 and 2 correspond to the residues that have been proposed to be involved in the substrate selectivity

O_2 versus NO reduction: enzymatic mechanisms

A major question in the field of the FDPs is whether these are oxygen or nitric oxide reductases, or both. In fact, for the few FDPs so far biochemically characterised, it appears that all three possibilities exist, although one should stress that comprehensive enzymatic studies leading to the determination of key kinetic parameters are scarce. In terms of electron donating capabilities, the fully electron loaded FDP (considering only the minimal core domain) has four electrons available (two in the FMN and two in the diiron centre) for the reduction of the oxygen molecule to water or for the reduction of two NO molecules to N_2O. An interesting outlier concerns the *E. coli* FDP, flavorubredoxin, whose one-electron reduced FMN (semiquinone) is kinetically stable. However, the extra rubredoxin domain may still provide the fourth reducing equivalent [41, 52].

Thus far, no significant differences were observed in the first coordination sphere for the centres from FDPs exhibiting different activities towards each substrate. The sole exception is the *D. gigas* FDP, where the conserved ligand His84 is replaced by a water molecule [10]. However, mutating in *Thermotoga maritima* FDP (with significantly



Scheme 1 Proposed mechanisms for NO and O_2 reduction by flavodiiron proteins adapted from [56, 57]

higher O_2 than NO reductase activity) the equivalent residue (His90) by Asn or Ala, Kurtz and co-workers observed no differences in the substrate preference, despite the replacement in the iron coordination of the His by Asn or a solvent molecule, respectively [53]. The available structural and functional data thus point to a modulation of the substrate selectivity by residues surrounding the diiron first coordination sphere (Fig. 6). Despite having essentially overlapping diiron centres both in terms of the iron ions and their ligands, the *G. intestinalis* FDP has a clear preference for O_2 [54], while the *Escherichia coli* FDP (flavorubredoxin) clearly favours NO reduction, both enzymes exhibiting a very low activity for the other substrate. An inspection of residues in the vicinity of the FDP diiron centres highlighted two notable differences. By generating single and double variants of *Entamoeba histolytica* EhFdp1 (an oxygen selective FDP) mimicking the residues found in the same positions in the NO selective *E. coli* FDP, we showed the relevant role played by two residues in modulating FDP enzymatic activity and substrate selectivity, particularly the Tyr271 residue in *E. histolytica* EhFdp1, which is replaced by a serine in *E. coli* FDP [44].

Regardless of the substrate specificity, the molecular mechanism for these enzymes has been comprehensively studied by Kurtz and co-workers, using the enzymes from *Thermotoga maritima* and *Treponema denticola* [55–59] (Scheme 1), employing a wide range of spectroscopic and kinetic methodologies. The proposed mechanisms for the reaction between reduced FDPs and NO involve the sequential formation of diferrous mononitrosyl and diferrous dinitrosyl intermediates, leading to the formation of N_2O. These studies contradicted previous proposals based on theoretical calculations involving the formation of a hyponitrite species [60]. The flavin was proposed by Kurtz and co-workers [55–57] to transfer electrons to the diferric site only after product release. Recently, the reaction between fully reduced FDPs and oxygen was proposed to involve the formation of a peroxo intermediate bridging the two ferric ions, without formation of intermediates with higher iron oxidation states [57].

Physiological functions of flavodiiron proteins

Most prokaryotic FDPs are generally considered to be cytoplasmic enzymes, due to the lack of signal peptides in their sequences. However, in cyanobacteria, which contain multiple copies of FDPs, some of them are proposed to be membrane-associated under certain conditions, namely close to photosystem II [27]. In eukaryotes, FDPs may be located in organelles: for example, in the unicellular protozoan *Trichomonas vaginalis*, one of the encoded FDPs is located in hydrogenosomes, which are organelles remnant of mitochondria metabolically adapted to anoxic life [40]; in the algae *Chlamydomonas reinnhardtii*, as probably in all eukaryotic oxygenic phototrophs, FDPs are located to the chloroplasts [61].

The first FDP to be isolated was proposed to be an oxygen reductase, which has in fact been later on firmly established for other organisms. Accordingly, the expression of FDPs is up-regulated by exposure of several anaerobic microbes to low oxygen levels (e.g., [43, 57, 62, 63]). A strong research line (led by E.-M. Aro and co-workers) within the FDP field concerns the role of cyanobacterial FDPs in protection of oxygenic photosynthesis, particularly by participating in oxygen photoreduction and protecting photosystems I and II under variable conditions, such as light intensity and CO_2 availability (reviewed in [14]). It is not a surprise that cyanobacteria, which produce O_2 as a byproduct of their photosynthetic metabolism, are particularly rich in FDPs, which afford a direct protection against oxygen. An important role for FDPs was also shown for

protection of the highly oxygen sensitive enzyme nitrogenase against oxygen, in *Anabaena* heterocysts [64].

In the enterobacteria *E. coli* and *Salmonella*, FDPs act as NO reductases, their expression being significantly upregulated in cell cultures exposed to authentic NO solutions or NO releasers under anaerobic conditions [11, 65–68]. For sulphate-reducing bacteria it was established that FDPs afford protection against NO under anaerobiosis, through the analysis of the phenotypes of FDP- null mutants [13, 16, 17].

The functions of FDPs may be particularly important in the context of host-microbe interactions, both from a host-pathogen strife viewpoint, or simply for survival of human microbiota within a challenging environment. For example, whereas the gut is generally considered an anaerobic milieu, oxygen concentrations can reach up to ~60 μM in the intestinal tract, particularly the colon [69]. On the other hand, NO is generated in the gut as a by-product of denitrification carried out by gut microbiota members and by acidification of nitrite. Therefore, the gut microbial population clearly benefits from O_2 and NO detoxification systems such as FDPs. Regarding the immune system weapons against invading microbes, mammalian macrophages attack pathogens initially through an intense oxidative burst, which is followed by the release of NO. Therefore, the resistance mechanisms that invading pathogens are endowed with are often considered virulence factors, since they constitute the first line of survival in the host's hostile environment. Indeed, a transcriptional analysis of virulent vs. non-virulent strains of the protozoan pathogen *E. histolytica* showed that an FDP-encoding gene has a significantly higher expression in the virulent HM-1:IMSS strain as compared to the non-virulent Rahman strain [70]. This observation, together with the O_2-regulation of *E.histolytica* EhFDP1 expression [43], underlines the important role of this enzyme as an oxygen defence system allowing this anaerobic pathogen to cope with varying oxygen tensions in the host gut. In *D. vulgaris,* FDP and, interestingly, one of its hybrid cluster proteins contribute to the survival of this bacterium in murine macrophages [71]. Concerning the enterobacterial NO-reducing FDPs, it has been demonstrated that FDPs have an important role in counteracting the nitrosative stress imposed by the host immune system [72–74].

Other O_2 and NO reductases

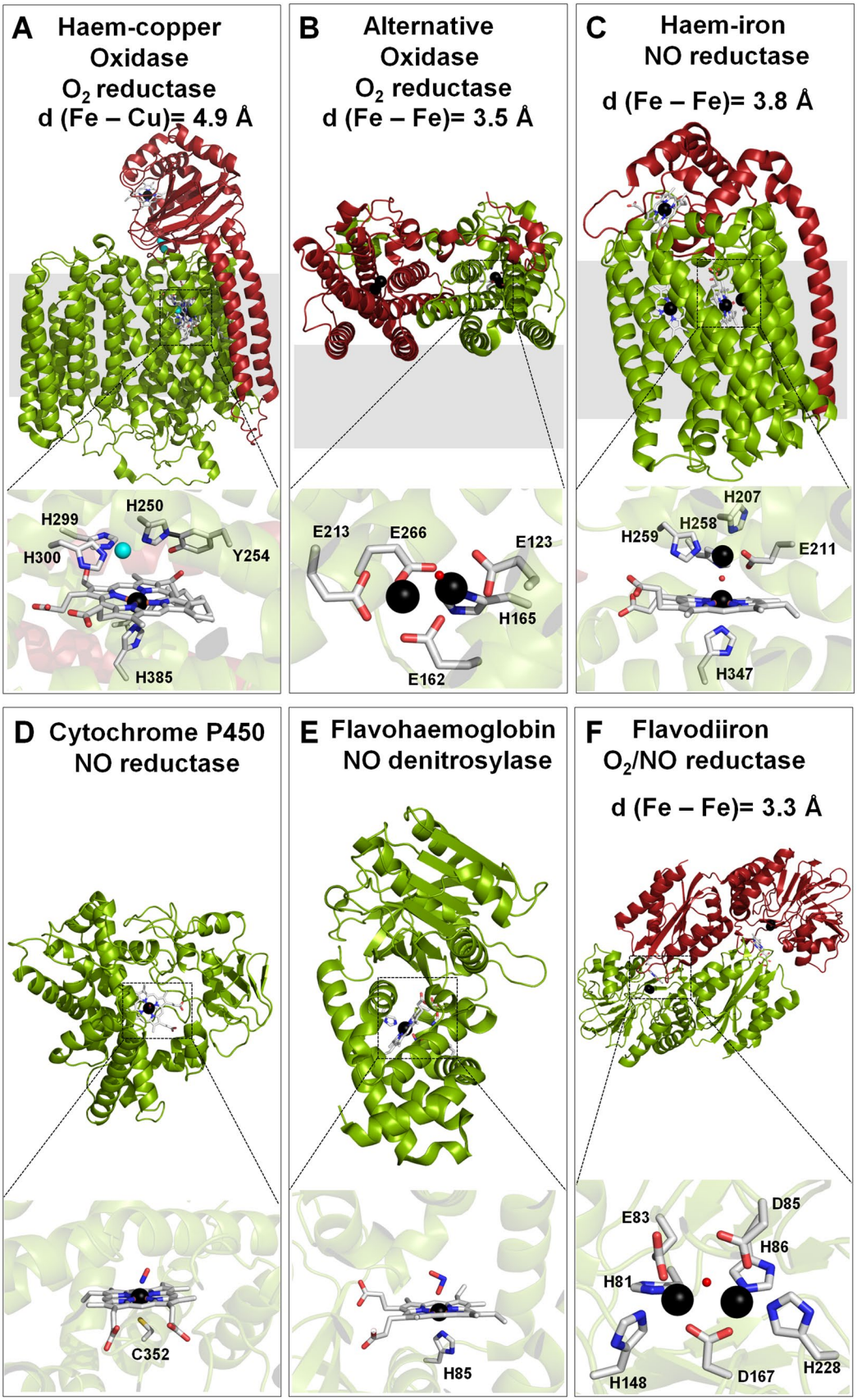
In the light of this special issue dedicated to R. J. P. Williams, it is interesting to compare FDPs with other enzymes having O_2 or NO reductase activities as their physiological function (Fig. 7), which illustrates the ingenuity and diversity of Nature encountered in the bioinorganic field. In fact, quite disparate enzymes perform similar functions in distinct biological contexts. For reduction of oxygen to water in aerobic respiration, there are three evolutionarily unrelated types of enzymes, all membrane-attached proteins: haem-copper oxygen reductases and cytochromes *bd,* both trans-membrane proteins, and alternative oxidases, that interact with the membrane surface via hydrophobic regions (Fig. 7).

Fig. 7 Cartoon representation of crystallographic structures of known O_2 or/and NO reductases. Oxygen reductases: **a** *Thermus thermophilus* haem-copper oxygen reductase (PDB code 2YEV), **b** *Trypanosoma brucei* alternative oxidase (PDB code 3VVA). NO reductases: **c** *Pseudomonas aeruginosa* haem-iron NO reductase (PDB code 3O0R), **d** *Fusarium oxysporum* cytochrome P450 NO reductase (PDB code 1CL6). **e** *Saccharomyces cerevisiae* flavohaemoglobin NO denitrosylase (PDB code 4G1V). **f** Flavodiiron proteins are represented according to their possible dual function by *Moorella thermoacetica* FDP (PDB code 1YCF). *Panels* indicate at the *top* the distance between the two metals and show cartoons of overall structures, using different colours for different subunits (when present); at the *bottom* it is shown a zoomed representation of the metal catalytic centre. Bonds are represented as *sticks*. μ(hydr)oxo-bridging oxygen moieties, iron and copper atoms are represented as *spheres* in *red*, *black* and *green*, repectively. *Gray shades* in **a**–**c** indicate the approximate location of the lipidic membrane bilayer

The haem-copper enzymes receive electrons from soluble metalloproteins or from quinols, and contain a binuclear catalytic centre built by a high-spin haem of the A or B types and a copper ion coordinated by three histidines. A possible μ-(hydr)oxo bridge connects the two metals in the oxidised state and one of the histidines coordinating the copper is covalently linked to a tyrosine. This residue plays a key role in the catalytic cycle by supplying an electron for the oxygen molecule splitting and reduction. Oxygen activation leads to the formation of ferryl intermediates (for recent reviews see [75, 76]), in contrast to what was recently proposed for the FDPs, which seem to avoid the formation of such high valent species (see above). In haem-copper oxygen reductases, the direct electron donor to the binuclear active site is a low-spin haem (also of A or B types) from the same subunit.

Cytochromes *bd* are quinol oxidases proposed to have as catalytic site a pair of haems B and D, although this remains to be fully clarified [77]. Besides oxygen reduction, cytochromes *bd* and haem-copper reductases have extra key functions: both are electrogenic and the latter also pump protons, i.e., both contribute to energy conservation through the build-up of a transmembrane difference of electric field, coupling the chemical (redox) reaction to charge translocation.

The alternative oxidase is a quinol:oxygen oxidoreductase containing an O_2-reducing diiron site embedded in a four-helix bundle motif, at the membrane surface, with a structure resembling that of rubrerythrins, a protein family involved in oxidative stress response [78, 79]. It had been proposed several years ago, prior to the knowledge


A Haem-copper Oxidase
O_2 reductase
d (Fe – Cu)= 4.9 Å
H299
H250
H300
Y254
H385
B Alternative Oxidase
O_2 reductase
d (Fe – Fe)= 3.5 Å
E213
E266
E123
H165
E162
C Haem-iron NO reductase
d (Fe – Fe)= 3.8 Å
H207
H258
H259
E211
H347
D Cytochrome P450 NO reductase
C352
E Flavohaemoglobin NO denitrosylase
H85
F Flavodiiron O_2/NO reductase
d (Fe – Fe)= 3.3 Å
E83
D85
H86
H81
H148
D167
H228

of the AOX crystallographic structure, that proteins of the rubrerythrin family (now proposed to act as hydrogen peroxide reductases (e.g., [80])) could have been the ancestors of AOX, as primitive oxygen reductases [81]. In the as-isolated enzyme the iron ions are coordinated only by carboxylates from glutamate residues, while the structures of the protein with an ascofuranone derivative or colletochlorin B show an additional imidazole ligand from His165 [78]. This change in iron coordination, resulting from the movement of one of the iron atoms, is reminiscent of the redox-linked ligand exchange observed in rubrerythrins [82, 83].

Both haem-copper oxygen reductases and cytochromes *bd* react with and are reversibly inhibited by nitric oxide in a complex way, depending on the relative concentrations of O_2, NO and reducing substrates. The haem-copper enzymes may form ferrous-haem nitrosyls or ferric-haem nitrite bound forms (in this case acting as NO oxidases), and some bacterial enzymes are endowed with low NO reductase activity [84–88].

The same type of variability is found in NO biochemistry. The respiratory membrane-bound NO reductases (NORs, involved in bacterial denitrification) are evolutionarily related to haem-copper oxygen reductases and belong to the same superfamily [89–92]. However, in respiratory NORs the binuclear centre where reduction of NO to N_2O occurs is formed by a haem B and a non-haem iron ion in place of the copper ion found in O_2 reductases; the iron ion is coordinated by three histidines and a glutamate [93] (Fig. 7). In line with R. J. P. Williams's ideas about evolution driven by element availability, the evolutionary relation between respiratory O_2 and NO reductases has been proposed to result from opposing iron and copper availability. Whereas the ancient anoxic atmosphere ensured the abundance of ferrous iron (highly soluble) and scarcity of copper (Cu^I is highly insoluble), the appearance of oxygen reverted this situation and the resulting oxidative environment favoured copper availability over iron, since Cu^{II} is highly soluble and ferric iron is highly insoluble. It has thus been suggested, yet disputed, that the haem-Fe NORs have preceded the appearance of the structurally related haem-copper oxygen reductases.

Another NO scavenging enzyme is the cytoplasmatic flavohaemoglobin, having as the catalytic site a high-spin haem B in a globin fold, which receives electrons from a flavin moiety in a ferredoxin-$NADP^+$ reductase (FNR)-like domain that oxidises NADH (e.g., [94]). Under aerobic or microaerobic conditions flavohaemoglobins oxidise NO to NO_3^-, in a denitrosylase reaction [95, 96]. However, in the absence of oxygen, flavohaemoglobins reduce NO to N_2O, through an as yet unknown mechanism, and have been shown to play an important role together with Class B FDPs in protecting *E. coli* from NO, under anaerobiosis [65].

Fungi have another completely distinct type of NO reductase, of the cytochrome P450 family (named P450nor), that is involved in denitrification. These enzymes are located in mitochondria or in the cytosol and do not exhibit any monooxygenase activity, but have the highest activity so far measured for an NO reductase, and use NAD(P)H as the direct electron donor [97]. The active site is a B-type haem coordinated to a cysteine thiolate, as in the superfamily of cytochromes P450, and the overall structure is basically the same as those of monooxygenase P450s [98]. The reaction with NO starts with the formation of a ferric-NO complex, possibly followed by a ferric-hydroxylamine radical complex, before the binding of a second NO molecule and N_2O release [98, 99].

The multihaem nitrite reductases reduce nitrite directly to ammonium and have a high-spin haem *c* as the catalytic centre, but are also able to reduce NO. Although this is not their primary function, these proteins may be important for NO detoxification under certain environmental conditions [100, 101].

Final remarks

A family of diiron containing enzymes, the flavodiiron proteins (FDPs), is now clearly established and found to be abundant in Archaea, Bacteria and Eukarya (particularly protozoa and oxygenic phototrophs), adding to the functional and structural diversity of proteins with histidine-carboxylate diiron centres. FDPs have a variable selectivity towards oxygen and/or nitric oxide, whose molecular basis is just beginning to be unravelled. FDPs are an example of the unpredictable variability of metalloenzymes, showing how the same catalytic function may be performed by completely distinct enzymes´ active metal centres, as well illustrated by the diversity encountered among other *bona fide* oxygen and nitric oxide reductases in different biological contexts. FDPs also show a quite interesting modular organisation, co-involving their redox partners, with structural domains similar to those acting in distinct enzymatic functions.

Flavodiiron proteins play key roles in the protection against oxidative or nitrosative stress, imposed by varying environmental conditions, including those created by the immune system. In cyanobacteria and algae, they are determinant for the protection of the photosynthetic apparatuses against oxygen, which suggests an early evolutionary appearance.

Just as the discovery of FDPs and their function was unexpected, so it may be anticipated that novel enzymes involved in oxygen or nitric oxide detoxification may be discovered in the near future, and evidences for alternative NO detoxification systems have already been proposed [66, 102].

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