Chapter 22
Flavodiiron Proteins and Their Role in Cyanobacteria

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22.1 Introduction

The flavodiiron proteins (FDP) constitute a large family of enzymes that reduce nitric oxide (nitrogen monoxide) to nitrous oxide (N_2O) or oxygen to water (for recent reviews see Saraiva et al. (2004); Vicente et al. (2008a–d)). These enzymes were given this name because they contain a flavin mononucleotide (FMN) and a diiron centre as the common prosthetic groups. The first reported example was *r*ubredoxin:*o*xygen *o*xidoreductase, ROO, from the sulphate reducing bacterium *Desulfovibrio* (*D.*) *gigas*. This enzyme was shown to be the terminal oxidase of a three-component electron transfer chain coupling NADH oxidation (by an NADH:rubredoxin oxidoreductase, NRO) to oxygen reduction (Chen et al. 1993a, b). Electron transfer from NRO to ROO is mediated by a small iron-protein, rubredoxin (Rd), which contains a FeCys4 centre (Chen et al. 1993b; Gomes et al. 1997). This process, which occurs in the cytoplasm, was proposed to confer oxygen tolerance to this anaerobic bacterium, simultaneously enabling NAD^+ regeneration (Fareleira et al. 2003). The first structure of an enzyme of the flavodiiron family was also that obtained for the *D. gigas* ROO, that allowed to clearly identify the catalytic centre, built by two iron ions ligated by the side chains of histidines and aspartates/glutamates (H^{79}-X-E^{81}-X-D^{83}-X_{62}-H^{146}-X_{18}-D^{165}-X_{60}-H^{226}, numbering of *D. gigas* ROO), and to envisage the intramolecular electron transfer pathways. Whereas the initial studies suggested a general role in oxygen stress alleviation in anaerobes, evidence for a role in nitric oxide detoxification broadened the interest on this protein family. In fact, nitric oxide is a well recognized toxic molecule, in concentrations above the nanomolar range, being either a weapon of the immune system or an intermediate of microbial denitrification; thus, organisms need to have enzymes able to deal with NO or its derived and toxic species. Until the discovery of this function of the FDPs, NO detoxification was thought to be mainly restricted

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to the flavohemoglobin family (for a recent review see Lewis et al. (2008)), while the heme-iron membrane-bound NO reductases are mainly considered as enzymes of the denitrification pathway.

Since those early findings, a wealth of data has been accumulated in this field for different organisms. The main features of this challenging enzyme family will be discussed, with a particular focus on what is so far known on flavodiiron enzymes from cyanobacteria.

22.2 FDPs: A Family of Modular Enzymes

The analysis of the primary structure of the large number of FDPs encoded in microbial and eukaryotic genomes revealed a common flavodiiron core with ca 400 amino acids, composed by a ~250-amino acids metallo-β-lactamase domain and a ~150-amino acids flavodoxin domain, and the presence of extra structural domains fused at the C-terminus of the flavodiiron core. This latter observation led us to establish four subfamilies (Classes A–D), according to the type of structural domains present in each protein (Fig. 22.1a–d); the domain arrangement is intimately associated with the type of electron transfer chains involved in each case (e.g., Saraiva et al. 2004; Vicente et al. 2008b).

The prototype enzymes, which constitute the bulk of identified FDPs, are those that have only the core domains, i.e., the β-lactamase and the flavodoxin-like domains (Class A, Fig. 22.1a). In several organisms these enzymes receive electrons from rubredoxins, acting as the terminal oxidase of a three components chain that involves also an NADH:rubredoxin oxidoreductase. However, in most organisms the immediate electron donor is not known and, as far as it can be predicted from genomic data, it is not a rubredoxin, since genes coding for this type of proteins are absent. Distinct types of electron donors have been reported, namely the $F_{420}H_2$ cofactor in methanogenic Archaea (Seedorf et al. 2007), and the pyruvate:ferredoxin oxidoreductase system for the *Trichomonas vaginalis* hydrogenosomal FDP (Smutna et al. 2009).

The second type of enzyme to be described was the so-called flavorubredoxin, FlRd, since it contains at the C-terminus a third structural domain, similar to rubredoxins. The ca 50 amino acids rubredoxin domain is fused to the flavodiiron core by an apparently unstructured linker of about 20 amino acids. So far, this type of enzymes (Class B, Fig. 22.1b) is restricted to enterobacteria, such as *Escherichia* and *Salmonella* species. FlRd has been shown to accept electrons directly from an NADH:FlRd oxidoreductase (from the family of NADH:rubredoxin oxidoreductases), establishing a two component electron transfer chain (Gomes et al. 2000, 2002; Vicente et al. 2007). The crystallographic structure of these enzymes remains to be determined, but structural studies by small angle X-ray scattering proposed that the linker between the Rd domain and the flavodiiron core is highly flexible, and thus the Rd domain protrudes into the solvent extending from the tetrameric quaternary structure, resembling an independently-behaving "quasi" free rubredoxin (Petoukhov et al. 2008).

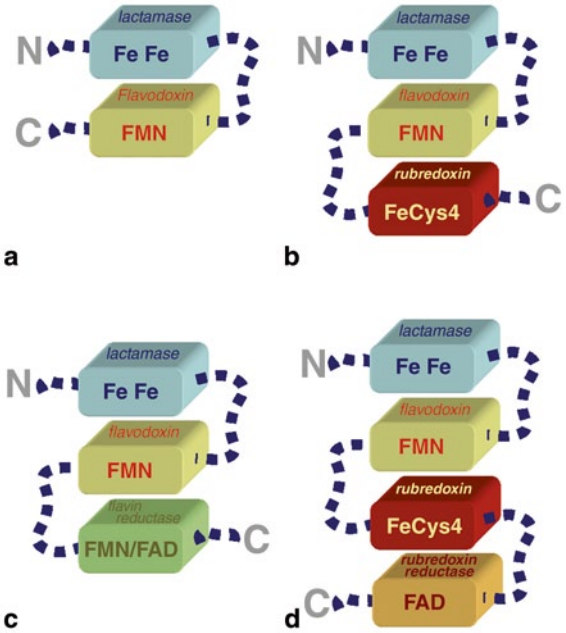

Fig. 22.1 Flavodiiron proteins are modular enzymes. Scheme depicting the modular nature of flavodiiron proteins, which in several organisms display extra C-terminal structural domains and were classified accordingly. ***a***—Class A FDPs are the structural prototype of this protein family, being composed of an N-terminal β lactamase-like domain (*light blue box*) and a C-terminal flavodoxin-like domain (*light yellow box*). ***b***—Class B FDPs, found thus far only in enterobacteria, bear an extra C-terminal rubredoxin domain (*dark red box*), harbouring a FeCys4 center. ***c***—Class C FDPs, from cyanobacteria and some eukaryotic oxygenic phototrophs, having a NAD(P)H:flavin oxidoreductase domain (*light green box*) fused at the C-terminus of the flavodiiron core, that may bind one FMN or one FAD moiety. ***d***—Class D FDPs are, so far, only found in the genomes of *Trichomonas vaginalis* and in a few *Clostridia* species. These FDPs appear to result from a fusion between a Class B FDP and its reductase partner, of the rubredoxin reductase family (*light orange box*)

Class C FDPs (Fig. 22.1c) were firstly identified in cyanobacteria (further discussed below)—these enzymes also bear a third domain at the C-terminus, homologous to NAD(P)H flavin reductases (Wasserfallen et al. 1998; Saraiva et al. 2004; Vicente et al. 2002, 2008a, b). Therefore, these FDPs have the interesting feature of condensing in a single polypeptide chain the whole electron transfer chain that couples NAD(P)H oxidation to oxygen and/or NO reduction.

The fourth class of FDPs (Class D, Fig. 22.1d) is composed by enzymes that appear to result from the fusion of a Class B FDP with the respective NADH:FlRd oxidoreductase, yielding a condensed four-domain polypeptide that is likely to directly accomplish O_2 or NO reduction at the expense of NAD(P)H oxidation. So far, the only examples were found in the genomes of the anaerobic protozoan *Trichomonas*

vaginalis (Carlton et al. 2007) and of the anaerobic bacterial pathogen *Clostridium perfringens* (Shimizu et al. 2002).

22.3 Amino Acid Sequences Analyses and FDPs Distribution

A BLAST search on the sequence databases using several queries (such as the sequences of the enzymes from cyanobacteria and from anaerobic bacteria) retrieved close to 500 sequences of flavodiiron proteins, which were aligned using ClustalX (Larkin et al. 2007). A dendrogram was constructed, based only on the common FDP core (discarding the extra C-terminal domains and several types of signal peptides at the N-terminus), using the neighbour joining method implemented in ClustalX (Fig. 22.2). FDPs, which were originally thought to be present only in prokaryotes, are also found in uni- and multicellular Eukarya, including oxygenic photosynthetic organisms (Table 22.1) and anaerobic protozoa (*Trichomonas, Giardia* and *Entamoeba* species). As previously proposed, there are evidences for multiple gene transfer events, but it is also possible to distinguish groups corresponding to particular phylogenies (Andersson et al. 2003, 2006; Vicente et al. 2008b). A striking example is the observation that sequences retrieved for the cyanobacteria and for all the other oxygenic photosynthetic organisms so far known to contain genes encoding FDPs (algae, mosses, lycophytes or even the higher plant *Picea sitchensis*, see Table 22.1) form a distinct clade from the other classes of FDPs. The sequences of 82 putative FDPs of cyanobacteria were aligned separately, together with those from eukaryotic oxygenic phototrophs (Table 22.1) and with a few other prototypic FDPs, which served as a reference; a subset of this alignment is presented in Fig. 22.3, and the resulting dendrogram in Fig. 22.2b. Genes coding for FDPs are present in all complete genomes of cyanobacteria, and each cyanobacterium sequenced has always at least two FDP encoding genes; cyanobacteria are also among the organisms that have a higher number of homologues in a single genome (up to six homologues, as in *Anabaena* sp. PCC7120 and *Anabaena variabilis* ATCC 29413, see Table 22.1). As already mentioned, all cyanobacterial FDPs and those from oxygenic phototrophs are of the C-type, i.e., have the extra C-terminal flavin-reductase domain; the only exception is the FDP from *Picea sitchensis*, which, in spite of having high amino acid identity/similarity with the homologues of the remaining photosynthetic organisms, lacks the flavin reductase domain. The fact that the FDP sequences of the eukaryotic oxygenic photosynthetic organisms are closer to the cyanobacterial ones than to those from other eukaryotes (see Fig. 22.2a, b), indicates that eukaryotes acquired these genes more than once, i.e., their origin is not monophyletic. It had already been proposed that the anaerobic protozoa had acquired FDPs from multiple lateral gene transfers from anaerobic prokaryotes (Andersson et al. 2003, 2006). Although a similar in-depth analysis is out of the context of this review, at least two arguments suggest a different scenario for the origin of the FDPs in oxygenic eukaryotes: (1) a close inspection of the sub-tree obtained

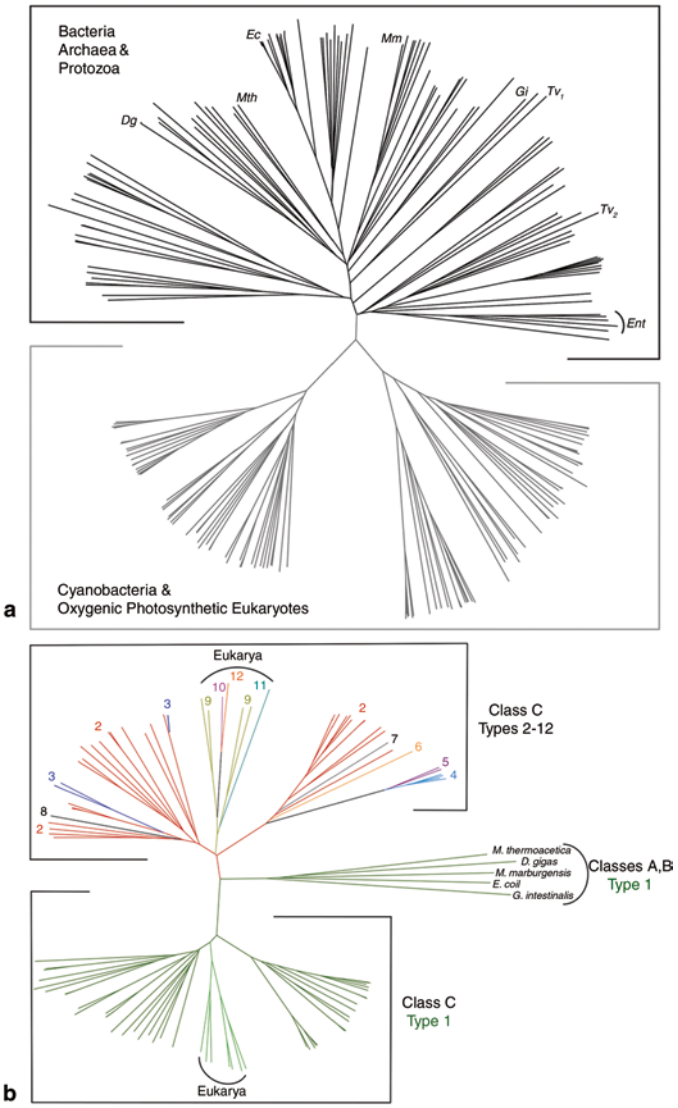

Fig. 22.2 **a** Dendrogram of selected flavodiiron proteins. A few sequences are highlighted: *D. gigas* (Dg), *Moorella thermoacetica* (Mth), *E. coli* (Ec), *Methanothermobacter marburgensis* (Mm), *Giardia intestinalis* (Gi), *Trichomonas vaginalis* (Tv_1, Tv_2), and several *Entamoeba* species (Ent). **b** Dendrogram of the flavodiiron proteins from cyanobacteria and oxygenic photosynthetic eukaryotes. The numbers correspond to the several Types of FDPs. Colors according to each type: *green*—1; *red*—2; *dark blue*—3; *light blue*—4; *magenta*—5; *light orange*—6; *gray*—7; *black*—8; *light green*—9; *pink*—10; *ocean blue*—11; *dark orange*—12. The dendrogram was obtained using ClustalX (Larkin et al. 2007), and Dendroscope, version 2.4 (Huson et al. 2007)

Table 22.1 Oxygenic photosynthetic organisms with genes coding for flavodiiron proteins

Organism	Order	No. of FDP genes
Cyanobacteria		
Acaryochloris marina (MBIC11017)	Acaryochloris	2
Anabaena (PCC7120)	Nostocales	6
Anabaena variabilis (ATCC 29413)	Nostocales	6
Cyanothece (ATCC 51142)	Chroococcales	4
Gloeobacter violaceus (PCC7421)	Gloeobacteria	2
Microcystis aeruginosa (NIES-843)	Chroococcales	4
Nostoc punctiforme (ATCC 29133)	Nostocales	5
Prochlorococcus marinus (MIT 9211, 9215, 9301, 9303, 9312, 9313, 9515; AS9601; NATL1A, 2A; MED4; SS120)	Prochlorales	2
Synechococcus (JA-2-3B′a(2-13); JA-3-3Ab; WH 7803, 8102; RCC307; CC9311, 9605, 9902, 9902; PCC7002)	Chroococcales	2
Synechococcus elongatus (PCC6301, 7942)	Chroococcales	2
Synechocystis (PCC6803)	Chroococcales	4
Thermosynechococcus elongatus (BP-1)	Chroococcales	2
Trichodesmium erythraeum (IMS101)	Oscillatoriales	2
Eukarya		
Algae		
Chlamydomonas reinhardtii	Chlamydomonadales	1
Micromonas pusilla (CCMP1545, RCC299)	Mamiellales	2
Ostreococcus lucimarinus (CCE9901)	Mamiellales	2
Ostreococcus tauri	Mamiellales	2
Paulinella chromatophora	Euglyphida	2
Lycophyte		
Selaginella moellendorffii	Selaginellale	2
Tree		
Picea sitchensis	Coniferales	1
Moss		
Physcomitrella patens (*subsp. Patens*)	Funariales	2

only for the oxygenic photosynthetic organisms (Fig. 22.2b) strongly suggests that the higher organisms obtained FDPs from an ancient, possibly non extant, cyanobacterium; (2) several putative FDPs of algae, mosses, lycophytes and of the tree *Picea sitchensis*, are nuclear encoded, and the deduced amino acid sequences reveal clear signal peptides at the N-terminus (peptides rich in aliphatic residues, as well as in basic residues, namely arginines (Gould et al. 2008)) which suggest that the FDPs will be present in cellular organelles. Quite interestingly, the FDPs of *Paulinella chromatophora* (an organism considered to have acquired its plastid from a recent endosymbiotic event that may have occurred only a few millions years ago (Bodyl et al. 2007; Yoon et al. 2009)), lack those signal peptides and, accordingly, are encoded in the plastidic genome. In a speculative way, it appears that migration

of the FDP genes from the plastidic chromosome to the nucleus did not yet occur in *Paulinella chromatophora*, contrary to what already happened quite longer ago in the other photosynthetic organisms. On this basis, as well as taking into account the proposed function for FDPs in cyanobacteria (see Sect. 22.7), it is tempting to suggest localization in the chloroplasts for the FDPs of the other eukaryotic phototrophs, but experimental work will be needed to prove this hypothesis. This situation is reminiscent of that for at least one of the FDPs of the protozoan *Trichomonas vaginalis*, which possesses a signal peptide targeting the enzyme to the hydrogenosome (Smutna et al. 2009).

As recently noted by E.M. Aro and co-workers (Zhang et al. 2009), the FDP sequences of the cyanobacteria form two distinct clusters (Fig. 22.2b), which those authors named A and B. However, due to the above mentioned classification of the FDPs according to the respective modular arrangement, we prefer a different nomenclature for this division. Indeed, a detailed analysis of the cyanobacterial sequences (c.f. Fig. 22.3) reveals another remarkable characteristic, as compared to the remaining FDP-encoding organisms: while about half of the sequences have conserved residues matching the "canonical" ones known to be involved in iron coordination (H^{81}-X-E^{83}-X-$D^{85}H^{86}$-X_{62}-H^{148}-X_{18}-D^{167}-X_{60}-H^{228}, *Morella thermoacetica* FDP numbering), which correspond to what we now propose to designate as Class C, Type 1 FDPs (Cluster B in Fig. 8 of Zhang et al. (2009)), a significant variation in these residues is present in the sequences forming the other cluster (Cluster A in Fig. 8 of that reference): up to eleven possible different ligand substitution combinations could be detected (Types 1–12, see Table 22.2, Figs. 22.2b and 22.3), with the corresponding sequences scattered along that second cluster, in between the second major group, the Type 2 enzymes. It is striking that the amino acid changes involve, in general, substitutions by neutral or positively charged residues (arginines, lysines and asparagines), or even aliphatic residues, such as isoleucines or alanines. It should also be noted that almost all cyanobacterial and photosynthetic eukaryotic organisms contain at least two FDP genes, one coding for a "canonical", Type 1 enzyme, and a second, encoding one of the multiple types identified (Tables 22.1, 22.2 and Fig. 22.3). Some sub-types appear to be more common than others (c.f. Table 22.2), and Types 9–12 are, so far, restricted to eukaryotes.

These multiple putative ligand substitutions will be further discussed in the next section, regarding the possible involvement of these amino acids as ligands for the iron ions. At present, no rationale can be proposed for this diversity and its apparent occurrence only in oxygenic phototrophs, either from the Bacteria or from the Eukarya domains.

The genomic organization of the genes encoding flavodiiron proteins is, in general, very diverse. In enterobacteria, the gene coding for the FDP forms a dicistronic unit with that coding its NADH oxidoreductase; in *D. gigas, roo* forms also a dicistronic unit with the gene encoding rubredoxin, the immediate electron donor to ROO. However, in the majority of the cases, the FDP-encoding genes have in their vicinity genes coding for proteins whose functions are unrelated to oxidative or nitrosative stress responses. We analysed the genome regions surrounding the 82

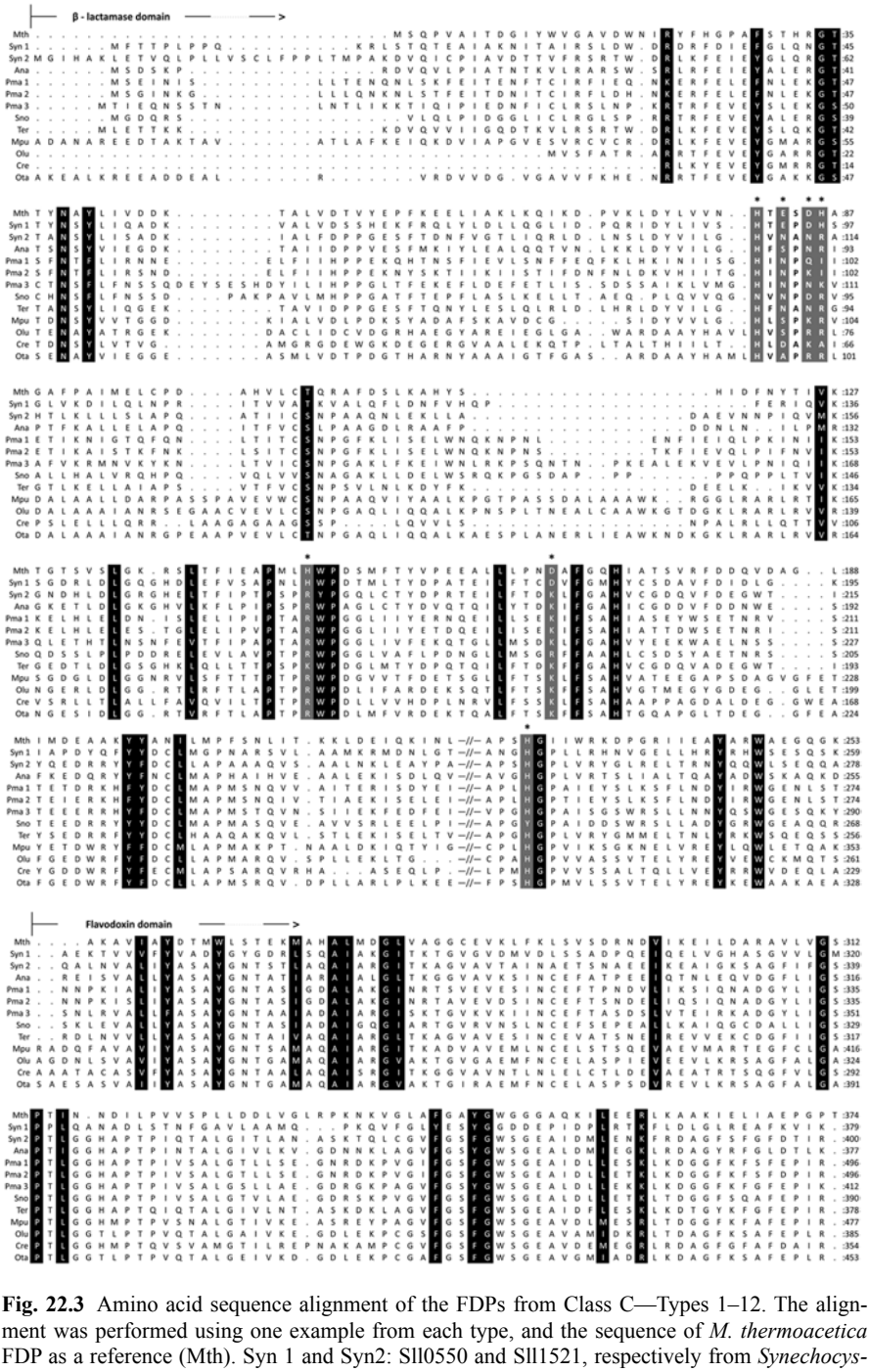

Fig. 22.3 Amino acid sequence alignment of the FDPs from Class C—Types 1–12. The alignment was performed using one example from each type, and the sequence of *M. thermoacetica* FDP as a reference (Mth). Syn 1 and Syn2: Sll0550 and Sll1521, respectively from *Synechocystis sp.* PCC6803; Ana: All0177 from *Anabaena sp.* PCC7120; Pma1: P9215_00501 from *Prochlorococcus* (*P.*) *marinus* MIT 9215; Pma2: P9515_00491 from *P. marinus* MIT 9515; Pma3: PMN2A_1375 from *P. marinus* NATL2A; Sno: SynRCC307_2387 from *Synechococcus* sp:

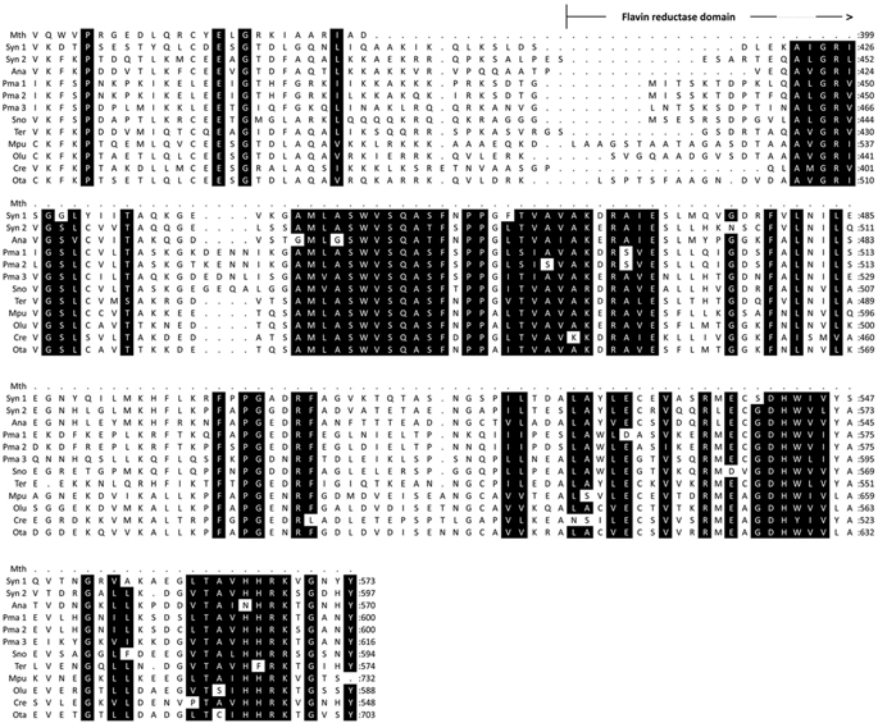

Fig. 22.3 (continued) RCC307; Ter: Tery_0770 from *Trichodesmium* erythraeum IMS101; Mpu: EEH_58658 from *Micromonas pusilla* CCMP1545; Olu: XP_001416100 from *Ostreococcus lucimarinus* CCE9901; Cre: XP_001692916 from *Chlamydomonas reinhardtii*; Ota: CAL52487 from *Ostreococcus tauri.* Several N-terminal extensions were deleted. The residues implicated in the binding of the diiron site in *Moorella thermoacetica* FDP are marked with an *asterisk* (*). *Black* shadows correspond to strictly conserved residues; *Gray* shadows correspond to the putative diiron site ligands. Too large non-conserved segments from the sequences designated by Mpu and Ota were removed for clarity and are represented by -//-

FDP-encoding genes retrieved from the Cyanobase website (http://genome.kazusa.or.jp/cyanobase/). From the 35 genomes scrutinized, 20 have genes coding for FDPs adjacently transcribed (Fig. 22.4a), where one of the genes corresponds always to a type 1 FDP, and the other to types 2–7. The flanking regions surrounding these contiguous FDP-encoding genes are variable. However, we found some examples of a common organization, as depicted in Fig. 22.4a. A particularly interesting case is found in a few *Prochlorococcus marinus* strains, where a rubrerythrin-encoding gene (a protein involved in oxidative stress response, as an H_2O_2 reductase (Kurtz 2006)) is adjacently upstream to one of the FDP coding genes and transcribed in the same direction.

In four genomes (including that of *Synechocystis* sp. PCC6803), two of the genes coding for FDPs are almost contiguous (always encoding a type 1 and a type 2 FDP enzymes), displaying only one gene (encoding a hypothetical protein) in between them, and the three genes are transcribed in the same direction (*sll0217-sll0219* in *Synechocystis* sp. PCC6803, Fig. 22.4b). That same gene is conserved among the

Table 22.2 Ligands of the diiron site in "canonical" FDPs, and corresponding amino acids substitutions in the enzymes from cyanobacteria and eukaryotic oxygenic phototrophs

Type (No. of examples)	Fe 1	Fe 2
1 (49)	H^{91}-X-E^{93}-X-D^{95}-H^{96}	H^{158}-X_{18}-D^{177}-X_{56}-H^{233}
2 (29)	H^{108}-X-N^{110}-X-N^{112}-R^{113}	R^{178}-X_{18}-K^{197}-X_{56}-H^{254}
3 (4)	H^{87}-X-S^{89}-X-N^{91}-R^{92}	R^{155}-X_{18}-K^{174}-X_{56}-H^{231}
4 (4)	H^{96}-X-N^{98}-X-Q^{100}-I^{101}	R^{174}-X_{18}-K^{193}-X_{56}-H^{250}
5 (2)	H^{96}-X-N^{98}-X-K^{100}-I^{101}	R^{174}-X_{18}-K^{193}-X_{56}-H^{250}
6 (1)	H^{105}-X-N^{107}-X-N^{109}-K^{110}	R^{190}-X_{18}-K^{209}-X_{56}-H^{226}
7 (1)	N^{89}-X-N^{91}-X-D^{93}-R^{94}	R^{168}-X_{18}-R^{187}-X_{56}-Y^{244}
8 (1)	H^{88}-X-N^{90}-X-N^{92}-R^{93}	K^{156}-X_{18}-K^{175}-X_{56}-H^{232}
9 (4)	H^{147}-X-S^{149}-X-K^{151}-R^{152}	R^{236}-X_{18}-K^{255}-X_{122}-H^{378}
10 (1)	H^{70}-X-S^{72}-X-R^{74}-R^{75}	R^{160}-X_{18}-K^{179}-X_{57}-H^{237}
11 (1)	H^{60}-X-D^{62}-X-K^{64}-A^{65}	R^{128}-X_{18}-K^{147}-X_{57}-H^{205}
12 (1)	R^{143}-X-A^{145}-X-R^{147}-R^{148}	R^{233}-X_{18}-K^{252}-X_{99}-H^{352}

Aminoacids numbering according to the sequences presented in Fig. 22.3, excluding *Morella thermoacetica* FDP. 1—*Synechocystis sp.* PCC6803 sll0550; 2—*Synechocystis sp.* PCC6803 sll1521; 3—*Anabaena sp.* PCC7120 all0177; 4—*Prochlorococcus* (*P.*) *marinus* MIT 9215 P9215_00501; 5—*P. marinus* MIT 9515 P9515_00491; 6—*P. Marinus* NATL2A PMN2A_1375; 7—*Synechococcus* sp RCC307 SynRCC307_2387; 8—*Trichodesmium* erythraeum IMS101 Tery_0770; 9—*Micromonas pusilla* CCMP1545 EEH_58658; 10—*Ostreococcus lucimarinus* CCE9901 XP_001416100; 11—*Chlamydomonas reinhardtii* XP_001692916; 12—*Ostreococcus tauri* CAL52487

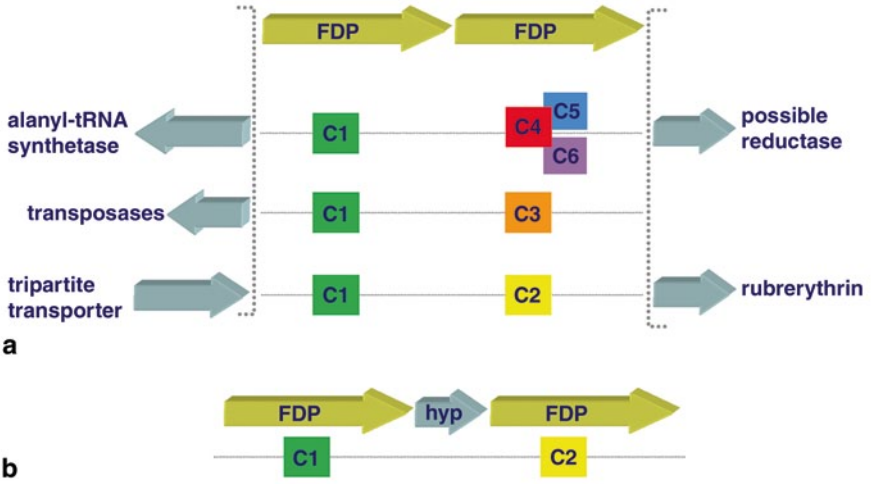

Fig. 22.4 Genomic regions surrounding cyanobacterial FDP encoding genes. The genome regions around each cyanobacterial FDP encoding gene were analyzed in the Cyanobase website (http://genome.kazusa.or.jp/cyanobase). **a** Types of genome loci that were found in at least two genomes, and the corresponding Type of Class C FDPs encoded. **b** In four genomes (including that of *Synechocystis* sp. PCC6803), genes encoding FDPs were found to be interspaced by a conserved gene encoding a hypothetical protein containing a transmembrane domain; these encoded FDPs are in every case a Type 1 and a Type 2

genomes displaying this organization, which suggests that transfer events may have included the set of FDP genes interspaced by this gene. Another interesting observation is that a secondary structure prediction (using the PSI-PRED server (Jones 1999, p. 839)) suggests the presence of transmembrane helices in that putative gene product. It should be recalled that the *Synechocystis* sp. PCC6803 *sll0217-sll0219* gene products (the type 1 and 2 FDPs in this kind of genomic organization) are found, at least partially, in the bacterial membranes, despite lacking any predictable transmembrane helices. Whether that hypothetical transmembranar protein contributes to this localization is an interesting question that requires experimental data to be answered.

22.4 Three Dimensional Structure of Flavodiiron Enzymes

The first step in the elucidation of the structural features of FDPs consisted in the resolution of the crystallographic structure of *D. gigas* FDP, rubredoxin:oxygen oxidoreductase (Frazão et al. 2000). This three dimensional structure confirmed the homodimeric quaternary arrangement determined in solution, with each ~43 kDa monomer being built by two structural domains: a diiron-containing domain, structurally analogous to zinc β-lactamases (despite the poor amino acids sequence similarity), followed by a short-chain flavodoxin-like domain, harbouring an FMN. In each monomer, the redox active cofactors are placed ca. 30 Å apart, precluding biologically active electron transfer between each other. However, the quaternary structure is such that, due to the "head-to-tail" arrangement of each monomer within the dimer, the diiron site of one monomer is almost in Van der Waals contact with the FMN from the other monomer, ensuring a fast electron transfer between the two redox centres (c.f. Fig. 22.5). The iron ions from the diiron centre are bound by histidines and glutamates/aspartates, and are bridged by a μ-hidroxo(oxo) species and one aspartate residue (Fig. 22.5c). Several FDP´s structures are now available (Silaghi-Dumitrescu et al. 2005; Seedorf et al. 2007; Di Matteo et al. 2008), revealing a conservation of the dimeric "head-to-tail" arrangement (even when the quaternary structure is a tetramer), as well as of the diiron ligand sphere.

As this revision was being written, the structure of a truncated form of *Anabaena* sp. PCC7120 FDP encoded by *all0177* (a Type 3 FDP), consisting solely of its lactamase-like domain was deposited in the Protein Data Bank (code 3HNN). The amino acids that act as iron ligands in "canonical", Type 1, FDPs, are herein replaced in several positions (Table 22.2); furthermore, the structure model does not contain any metal ion. Whereas the overall fold of this truncated domain resembles the correspondent ones in the other FDPs (Fig. 22.5a), the structure of the diiron site pocket is markedly different (Fig. 22.5d). In this structure, the side chain of a lysine (K174, all0177 numbering, equivalent to K197 in Fig. 22.5d—see sequence alignment in Fig. 22.3), which replaces the bridging aspartate in Type 1 FDPs, protrudes into the space occupied by the iron ions in the other FDPs. Moreover, the number of basic

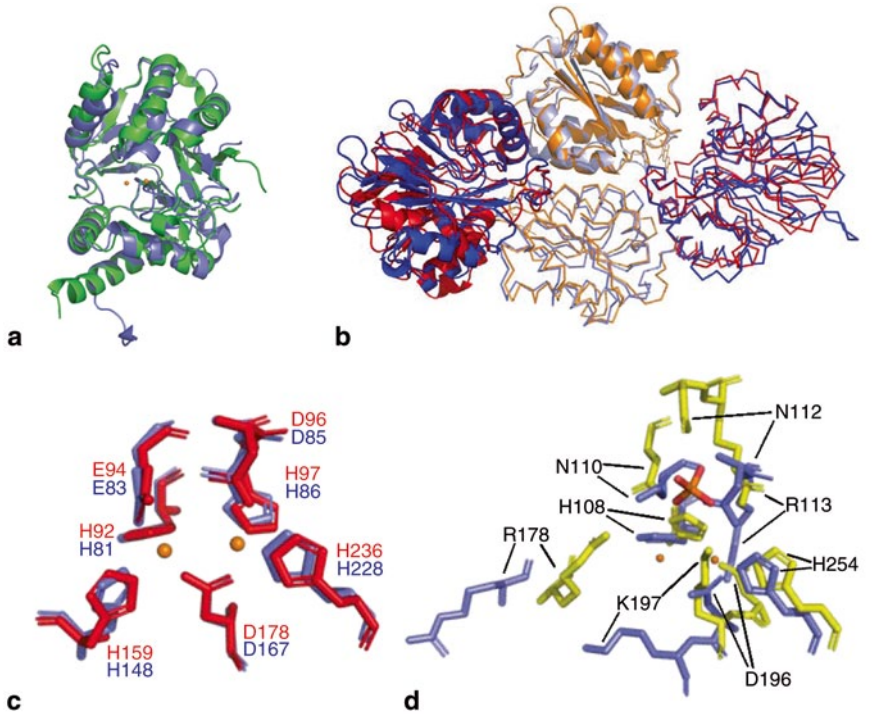

Fig. 22.5 Structural models of cyanobacterial flavodiiron proteins. **a** Structures of β-lactamase domains of a Class A FDP (*Moorella thermoacetica* FDP, PDB code 1YCG, in *blue*), and of a Class C Type 3 FDP (*Anabaena* sp. PCC7120 all0177, PDB code 3HNN, in *green*). The structures were aligned in Pymol (DeLano 2002) with an RMS of 1.364 Å for 185 aligned residues. **b** Models of the flavodiiron core of cyanobacterial FDPs (the C-terminal flavin reductase domains were removed from the modelled sequences). Two types of *Synechocystis* sp. PCC6803 FDPs were modelled using deposited FDPs structures as templates. sll550 (Type 1 FDP) was modelled using 1E5D, 1VME and 2OHH as templates (model in *blue*); sll1521 (Type 2 FDP) represented in *red* and *orange*, modelled using 2OHH as template. Each monomer is displayed either in cartoon or ribbon representation. **c** Superimposed modelled diiron site of sll550 (in *blue*) and of *Moorella thermoacetica* FDP (in *red*); iron ions as *orange* spheres. **d** Superimposition of the modelled diiron sites of sll1521 (Type 2), using different modelling strategies: *blue,* ligand substitutions modelled with structures of "canonical" Class A FDPs structures; *yellow,* the same "substituting" residues modelled with the structure of *Anabaena* sp. PCC7120 Type 3 FDP lactamase domain (PDB code 3HNN)

residues, such as asparagine and arginine (that replace the "canonical" carboxylate ligands) crowding the pocket may be the reason why a phosphate ion is stabilized inside the cavity. Since this structure was obtained for a truncated domain, one has to take into account a possible destabilization of the overall structure, in spite of the apparently retention of the lactamase fold (as described above and shown in Fig. 22.5a). This raises the question whether iron is missing because of the nature of the "alternative ligands", or due to any technical issue related with the protein production, purifi-

cation or crystallization processes, which may trigger a rearrangement of the binding pocket. It should be noted that in several of the other types of enzymes (Types 2–8) the residue that replaces the bridging aspartate is preceded by another aspartate or by a glutamate, which may fulfil the same role, as will be discussed below. However, from the amino acid sequences comparison (see data on Table 22.2 and Fig. 22.3), it is clear that while some amino acid substitutions are conservative, in the sense that the amino acid substitute may bind to a metal ion through its side chain (even if representing less frequent modes of biological metal coordination), other amino acids may bind only through the backbone carbonyls, as the respective side chains are aliphatic (for example, the cases of isoleucine and alanine). Also, in many cases it is not possible to find in the sequences possible sensible amino acid substitutes, being the bridging aspartate a possible exception to this observation. These multiple amino acid substitutions raise the question whether some of those "unusual" FDPs are indeed metalloenzymes, which may be clarified only by appropriate experimental data. Of course, the possible absence of the iron ions will have unpredictable influences on the protein function.

Physiological studies on cyanobacterial FDPs have thus far focused on *Synechocystis* sp. PCC6803 enzymes (Vicente et al. 2002; Helman et al. 2003; Hackenberg et al. 2009; Zhang et al. 2009). This organism's genome codes for four FDP homologues, two of which fall into Type 1 and the other two into Type 2 subgroups. Since there appears to be no difference in terms of physiological roles between the two types of FDPs (see Sect. 22.7), we attempted to predict the structures of one of each type using deposited PDB files as templates. Since Class C FDPs have the extra C-terminal flavin reductase domain, the sequences used for modelling the structure were previously truncated, in order to model only the flavodiiron core. The protein coded by gene *sll0550* (a Type 1 enzyme) was modelled by combining the generated optimal structural alignments between sequence segments and several FDP structures (*D. gigas* ROO (1E5D), *Thermotoga maritima* FDP (1VME) and *Methanothermobacter marburgensis* $F_{420}H_2$ oxidase (2OHH)). The resulting model is thus an assembly of the best-fit models for different parts of the sll0550 sequence. The predicted structure (Fig. 22.5b) is quite similar to those of the "canonical" FDPs. Looking in detail at the binuclear site (Fig. 22.5c, red), it is observed that the conserved diiron ligands are modelled in the same positions and geometries as those of *Moorella thermoacetica* FDP (Fig. 22.5c, blue).

The Type 2 FDP, encoded by *sll1521*, was modelled by two different strategies. First, it was modelled using as template the structure of *Methanothermobacter marburgensis* $F_{420}H_2$ oxidase (2OHH). By this approach, it was possible to obtain a model where the residues substituting the "canonical" ligands display a geometry compatible with an iron coordination equivalent to Type 1 FDPs (Fig. 22.5d). Moreover, this modelling strategy resulted for sll1521 in a structure where the iron ions are present and the aspartate preceding the substituting lysine is placed in the same three dimensional position as the "canonical" aspartate. This model for sll1521 is thus compatible with a protein where the alternative amino acids indeed bind the iron ions. However, when we modelled sll1521 using the structure of the lactamase-like domain of *Anabaena* sp. PCC7120 Type 3 FDP (PDB code 3HNN) as a template, the structure of the diiron binding pocket changed dramatically, as expected

(Fig. 22.5d). As already noted for the *Anabaena* truncated enzyme, the side chain of the lysine that substitutes in the sequence of all177 the bridging aspartate protrudes into the space occupied by iron in Type 1 FDPs. Moreover, the equivalent basic residues that substitute the Type 1 ligands are placed in the same position as those in the al177 structure. Therefore, the fact that both models are plausible leaves an open question, whether the metal ions that constitute the diiron site are present or not in the native sll1521 enzyme, and which would be the effect of their presence or absence on the protein's function. As above mentioned, this question may be answered only with further structural and functional studies of the different types of FDPs.

22.5 Biochemical Properties

The FDPs thus far characterized are homodimers or homotetramers (a dimer of "head-to-tail" homodimers) in solution. Each monomer has ~45 kDa (Class A), ~54 kDa (Class B), and ~63 kDa (Class C) (no Class D FDP has been characterized), and contains two iron ions and one FMN per flavodiiron core, plus one iron per rubredoxin domain in Class B FDPs, and one flavin per flavin reductase domain in Class C FDPs. The latter extra domain is promiscuous in terms of flavin, being capable of harbouring FMN or FAD (Vicente et al. 2002). Due to the very low molar absortivity of the diiron centres, the electronic spectra of these enzymes are dominated by the flavin absorption, with maxima at ~460 and ~380 nm. In Class B enzymes, due to the presence of the FeCys4 centre the band at ~460 nm shifts to ~470 nm and an extra band appears at ~570 nm. Most Class A flavodiiron proteins thus far characterized displayed visible spectra which were broad and smooth in the band centred at ~460 nm. However, the spectra of a cyanobacterial FDP (Synechocystis sp. PCC6803 sll0550 (Vicente et al. 2002)) and of a methanogenic FDP (Wasserfallen et al. 1995) display this band with two shoulders. It has been noted that the FDPs with this kind of spectrum, lack a conserved tryptophan residue in the flavodoxin-like domain (Trp347 in *D. gigas* ROO numbering) which is coplanar with the FMN isoalloxazine ring. It was thus proposed that the presence/absence of this Trp residue was responsible for the heterogeneity in the spectral shape of the flavin moiety (Saraiva et al. 2004).

The diiron centre was first assessed by the structure of the *D. gigas* enzyme (Frazão et al. 2000). Later, it was studied in detail by EPR spectroscopy for the *E. coli* enzyme (Vicente and Teixeira 2005), and more recently for the *Giardia* and *Thrichomonas* enzymes (Vicente et al. 2009; Smutna et al. 2009). As characteristic of this type of iron centre, it can exist in three different oxidation states: diferric (Fe(III) Fe(III)), mixed valence (Fe(III) Fe(II)) and diferrous (Fe(II) Fe(II)). The diferric state is EPR silent, since the two iron ions (high-spin, $S=5/2$) are antiferromagnetically coupled, yielding a total spin $S=0$; for the *E. coli* enzyme a resonance at $g\sim11$ for the diferrous state was detected, suggesting a $S=4$ spin ground state. The mixed valence state has a total spin $S=1/2$ and is EPR active, yielding a typical set of resonances below $g=2.0$. The diiron centre of *Moorella thermoacetica* FDP was characterized by Mössbauer spectroscopy, confirming that

the iron ions in the binuclear site are magnetically coupled (Silaghi-Dumitrescu et al. 2003).

The spectroscopic signatures from the several redox cofactors of FDPs were used to study their redox properties, combining Visible and EPR spectroscopies with potentiometric titrations. The flavodoxin domain-bound FMN cofactor of several FDPs displayed reduction potentials in the −224 to +25 mV range for the $FMN_{ox} \rightarrow FMN_{sq}$ transition and −117 to +25 mV range for the $FMN_{sq} \rightarrow FMN_{red}$ transition (Gomes et al. 1997; Silaghi-Dumitrescu et al. 2003; Vicente and Teixeira 2005; Vicente et al. 2009; Smutna et al. 2009). The majority of the FDPs studied have reduction potentials that are enough separated to allow transient stabilization of the one electron-reduced semiquinone (Sq) state, which so far is always of the red, anionic type. However, recent studies on protozoan FDPs reported very close reduction potentials for the two redox transitions of the flavin, suggesting that the semiquinone state is not stabilized in these enzymes. Notably, those enzymes are only able to efficiently reduce oxygen, whereas the other FDPs, which stabilize the semiquinone flavin state, are either only NO (Vicente and Teixeira 2005) or ambivalent NO/O_2 reductases (Silaghi-Dumitrescu et al. 2003; Rodrigues et al. 2006). Until now, it has not been possible to propose a rationale for this difference in redox behaviour between O_2-reducing and NO/O_2-reducing FDPs, mainly because, at the available structural resolutions, the flavin-binding pockets are essentially identical among the several FDPs. Concerning the binuclear iron active site, the reduction potentials have been determined only for the *E. coli* flavorubredoxin (Vicente and Teixeira 2005) and for the protozoan FDPs (of *Trichomonas vaginalis* and *Giardia intestinalis*) (Di Matteo et al. 2008; Smutna et al. 2009), coupling redox potentiometry to EPR spectroscopy. The diiron sites of the protozoan enzymes have much higher reduction potentials than those of the *E. coli* FDP. However, whereas the reduction potentials in all three cases are compatible with oxygen or nitric oxide reduction, it is noteworthy that the two protozoan enzymes, that display only O_2 reductase activity, have reduction potentials markedly different from those of the NO-reducing *E. coli* enzyme. As mentioned for the flavin cofactor, the available structures do not allow yet understanding the differences in redox properties of the diiron centers.

One interesting aspect of FDPs redox properties is the observation, for the *E. coli* FlRd, that the reduction potentials of the iron center are altered in the presence of the reductase partner, which strongly suggests the formation of an electron transfer complex between the two enzymes (Vicente and Teixeira 2005).

22.6 Enzymatic Studies

Enzymatic studies to assess the putative role of FDPs as oxygen or nitric oxide reductases have been performed in Clark-type electrodes specific for each molecule. In typical experiments, *in vitro* electron transfer chains were assembled to medi-

ate, under non rate-limiting conditions, electron delivery from NAD(P)H to FDP and determine its reductase activity towards each substrate. Fast kinetics methods, namely stopped-flow coupled to Visible spectroscopy was also used to further confirm the steady-state activity of the *Giardia* enzyme (Di Matteo et al. 2008).

The first enzymatic studies on a member of this protein family, performed for *D. gigas* ROO, led to the proposal that FDPs would be involved in the detoxification of dioxygen, which is both an inhibitor of many enzymes from anaerobes and a source of deleterious reactive oxygen species. Following the initial characterization of the *E. coli* enzyme that was shown to be capable of binding nitric oxide (Gomes et al. 2000), Gardner and co-workers proposed that *E. coli* FlRd was involved in NO detoxification under anaerobic conditions (Gardner et al. 2002). This proposal led to the *in vitro* demonstration of nitric oxide reduction by FlRd, coupled to NADH oxidation by its physiological partner, the NADH:FlRd oxidoreductase, with a turnover of 15–20 s^{-1} (Gomes et al. 2002). Since then, nitric oxide reductase activities of the same order of magnitude have been determined for several other FDPs (Gomes et al. 2002; Silaghi-Dumitrescu et al. 2003, 2005; Rodrigues et al. 2006). However, the data so far available indicate that the FDPs may have an ambivalent activity, i.e., some are able to reduce mainly NO, others mainly, or even exclusively, O_2 (Di Matteo et al. 2008; Smutna et al. 2009), and there are few cases for which comparable activities with both substrates were measured (Silaghi-Dumitrescu et al. 2003, 2005; Rodrigues et al. 2006; Hillmann et al. 2009). Concerning *Synechocystis* sp. PCC6803, preliminary data obtained for Sll0550 revealed that the enzyme has a relatively low oxygen reductase activity, which could have been hampered by the sub-stoichiometric flavin load (Vicente et al. 2002). It is important to emphasize that the data available also suggest a sluggish reaction of the reduced enzymes with hydrogen peroxide, H_2O_2, and with several reactive nitrogen species (Di Matteo et al. 2008; Smutna et al. 2009 and our own unpublished data), i.e., the enzymes appear to favour NO and O_2 as substrates. In this respect, it should be mentioned that the substrate binding cavity present in zinc β-lactamases is occluded in FDPS (e.g., Frazão et al. 2000). The dual oxygen/nitric oxide reducing activity raises the question whether both activities are physiologically relevant and what dictates the preference for each substrate. The evidences for the physiological roles of FDPs will be further discussed in the next section.

22.7 Physiological Roles of FDPs: FDPS as O_2 and/or NO Reductases

The work by Gardner and co-workers first showed that FlRd protects *E. coli* against nitric oxide; deletion of the *flrd* gene highly compromised the growth viability of *E. coli* upon exposure to NO, under anaerobic conditions (Gardner et al. 2002). Furthermore, it was shown that *flrd* transcription is regulated by NO via NorR (Gardner et al. 2003), a transcriptional regulator that in enterobacteria is located immediately upstream, and divergently transcribed of the dicistronic unit that en-

codes both FlRd and its NADH oxidoreductase (da Costa et al. 2003). Saraiva and co-workers (Justino et al. 2005a) further showed that the binding of the NorR trimer to three sites of the flavorubredoxin gene promoter is required for nitric oxide-dependent induction of *flrd.* Several transcriptomic studies confirmed that *flrd* transcription is indeed up-regulated by NO (Mukhopadhyay et al. 2004; Flatley et al. 2005; Justino et al. 2005b; Pullan et al. 2006), including in *E. coli* cells grown under anaerobic conditions (Justino et al. 2005b). On the contrary, transcriptional studies performed for *E. coli* under several oxidative stress conditions did not reveal a significant alteration on the expression of the FlRd-encoding gene. These *in vitro* studies established a role for flavorubredoxin (a Class B FDP) in *E. coli,* as an enzyme responsive to the deleterious action of nitric oxide, namely under anaerobic conditions. Similar evidences were later on obtained for a few other bacteria, such as *D. gigas* (Rodrigues et al. 2006). But, in apparent agreement with the fact that the *D. gigas* enzyme has comparable NO and O_2 reducing activities, ROO seems to be important also under oxidative stress conditions (Rodrigues et al. 2006). A similar situation was recently reported for *Clostridium acetobutylicum*, in which the flavodiiron proteins were shown to be up-regulated upon exposure of *C. acetobutylicum* to O_2 and important to protect this anaerobic bacterium against oxygen (Hillmann et al. 2009).

For cyanobacteria, there is no data regarding the role of FDPs in response to nitrosative stress conditions; however, as will be described below, several experimental data clearly suggest a function for their FDPs in oxidative stress protection.

A survey of DNA microarray data so far reported for *Synechocystis* sp. showed that the genes encoding FDPs have their expression modified under certain conditions. The expression of the already mentioned gene cluster *sll0217-0219* was found to be induced during *Synechocystis* acclimation from low- to high-light intensity (Hihara et al. 2001). The *sll0217* gene is also among the 36 genes encoding potential FMN-containing proteins that were induced by UV-B light (Huang et al. 2002). The induction of the FDP encoding genes observed under high energy supply is possibly related to the higher generation of reactive oxygen species under these conditions; in fact, other genes encoding ROS scavenging enzymes, such as superoxide dismutase and glutathione peroxidase, were also up-regulated under those conditions (Huang et al. 2002). Chauvat and co-workers (Houot et al. 2007) reported that the transcription of the *sll1521, sll0217* and *sll0219* genes, encoding FDPs, increases in *Synechocystis* cells exposed to 3 mM hydrogen peroxide, or grown in the presence of excess of metals such as cadmium, iron and zinc. Nevertheless, the observed variations were low (2-4-fold) and contradictory data has been reported (Hihara et al. 2003; Kobayashi et al. 2004; Li et al. 2004). In summary, the DNA array data is still too scarce and somehow ambiguous. More specific and clarifying studies were performed also for *Synechocystis* sp. PCC6803, mainly based on the analysis of the behaviour of several single and multiple deletion mutants of its FDP genes under different growth conditions, namely illumination and carbon dioxide fluxes, and combinations of both these factors (Helman et al. 2003; Hackenberg et al. 2009; Zhang et al. 2009). It was concluded that sll1521 and sll0550 (enzymes of Types 2 and 1, respectively) were essential for the Mehler reaction, i.e., for elec-

tron transport from photosystem I to oxygen, without formation of reactive oxygen species. In the same report it was proposed that the two enzymes could assemble as a heterodimer, and that sll0550 could be sufficient to sustain photoreduction of dioxygen. More recently was studied in further detail the possible role of the other two FDPs, sll0217 and sll0219 (Zhang et al. 2009). The main conclusion was that both enzymes were important to protect photosystem II against oxidative stress, caused by conditions of high illumination intensity and/or low carbon dioxide fluxes. Interestingly, it was further shown that those FDPs are membrane associated, and that the expression of *sll0217* was enhanced in the single deletion mutants of *sll1521* or *sll0550*, i.e., it appears that sll0217 may substitute also for either of those two enzymes.

These proposals are in line with an oxygen reductase activity of the cyanobacterial enzymes, as first shown for the gene product of *sll0550* (Vicente et al. 2002): the depletion of dioxygen by a NAD(P)H:oxygen oxidoreductase activity of the FDPs is fully consistent with their proposed roles in acting against oxidative stressing conditions, avoiding the formation of reactive oxygen species through the direct reduction of oxygen to water and enabling the elimination of excess reducing equivalents in the photosynthetic electron transfer chain. Nevertheless, these activities remain to be directly proven *in vitro* for the other three *Synechocystis* FDPs. This is particularly relevant, due to the fact that there is no apparent correlation between the proposed functions for the *Synechocystis* enzymes and their types, i.e., the presence or absence of the "canonical" ligands: sll1521 and sll0219 are enzymes of Type 2, lacking some of those ligands, while sll0550 and sll0217 are enzymes of the "canonical" Type 1.

22.8 Concluding Remarks

In summary, the flavodiiron proteins are a family of modular enzymes having nitric oxide and/or oxygen reductase activities. Both activities are supported either by *in vitro* experiments or by *in vivo* approaches, namely through transcriptional and phenotypic analysis of wild type and deletion strains. The abundance of homologues in cyanobacteria is particularly striking and may be related to the fact that oxygenic phototrophs are particularly prone to oxidative stress, due in special to the endogenous production of dioxygen and to the delicate balance between photonic energy supply and carbon dioxide availability. As flavodiiron enzymes appear to have evolved quite early, eventually before the split between Bacteria and Archaea, they may have been already present when oxygenic photosynthesis started, ca 3–3.5 billion years ago, as one of the first mechanisms to respond to the "oxygen paradox", i.e., to avoid its intracellular toxicity. A similar scenario has been proposed for another type of diiron proteins, the rubrerythrins (Gomes et al. 2001), which more recently were proposed to act as hydrogen peroxide reductases, forming water directly through reduction of H_2O_2 (e.g., Kurtz 2006) and which are also quite spread among cyanobacteria (our unpublished observation). A schematic representation of

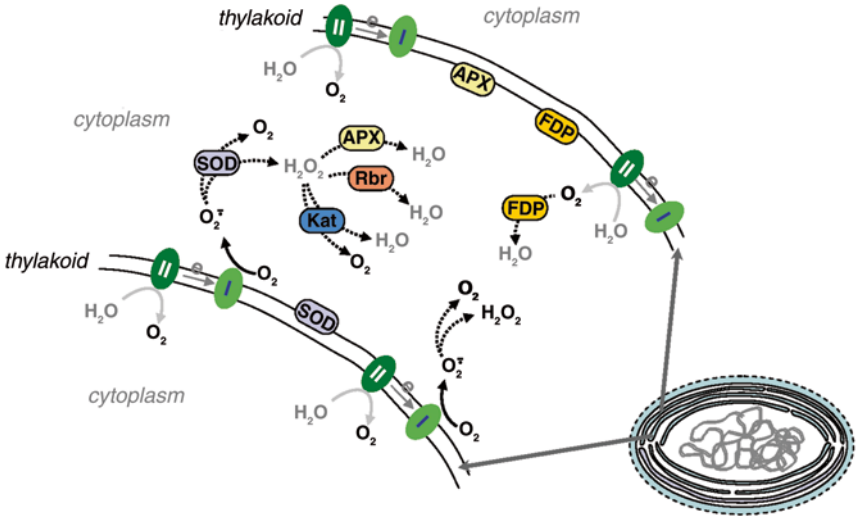

Fig. 22.6 Scheme illustrating oxidative stress and response mechanisms in cyanobacteria. Zoom-in into the thylakoid membranes in cyanobacteria and the cytoplasmatic space in between, where formation of reactive oxygen species and their breakdown by specialized enzymes are likely to take place. Oxygen is produced by water oxidation at the oxygen-evolving complex in Photosystem II (PS II, in *dark green*). Excess electrons in Photosystem I (PS I, in *light green*) generate superoxide anion from reaction with molecular oxygen; superoxide can self-dismutate to yield oxygen and hydrogen peroxide. Excess oxygen can be fully reduced to water by flavodiiron proteins (FDP, in *orange*), soluble and possibly also membrane-associated. Besides its self-dismutation, superoxide anion can be efficiently scavenged by superoxide dismutase (SOD, in *light blue*), both soluble and membrane-bound, whose products are O_2 and H_2O_2. Hydrogen peroxide can be removed by at least three different enzymatic systems detected in the genomes of cyanobacteria: both ascorbate peroxidase (APX, in *yellow*, soluble and membrane-bound forms) and rubrerythrin (Rbr, in *red*) can reduce hydrogen peroxide to water; catalase (Kat, in *darker blue*) generates oxygen and water

the diverse mechanisms involved in oxidative stress protection in cyanobacteria is presented in Fig. 22.6, where it becomes clear the importance of having enzymes, such as FDPs, that avoid the production of reactive oxygen species, like the superoxide anion (detoxified by superoxide dismutases), or hydrogen peroxide (scavenged by catalases, peroxidases, rubrerythrins or ascorbate peroxidases). It has still to be shown if in cyanobacteria FDPs play also a role in nitrosative stress defence. But whatever the actual function of the several homologues of FDPs will be, their multiplicity suggests a crucial role in cyanobacteria.

Several challenging questions remain to be answered: one relates to the evolution of FDPs and their function in higher eukaryotes; the second, the unusual diversity of putative "ligands" to the diiron site—do all types of FDPs here described contain indeed a metal site? If yes, they will have quite unprecedented ligand combinations; if no, which will then be the function of the non-metal containing enzymes? Answers to these questions will bring new insights into the structure and function of diiron

proteins and into the field of oxidative stress, and will further enlarge the knowledge on the physiology of oxygenic phototrophs.

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