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REVIEW

Ferredoxin: the central hub connecting photosystem I to cellular metabolism

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Abstract

Ferredoxin (Fd) is a small soluble iron-sulfur protein essential in almost all oxygenic photosynthetic organisms. It contains a single [2Fe-2S] cluster coordinated by four cysteine ligands. It accepts electrons from the stromal surface of PSI and facilitates transfer to a myriad of acceptors involved in diverse metabolic processes, including generation of NADPH *via* Fd-NADP-reductase, cyclic electron transport for ATP synthesis, nitrate reduction, nitrite reductase, sulfite reduction, hydrogenase and other reductive reactions. Fd serves as the central hub for these diverse cellular reactions and is integral to complex cellular metabolic networks. We describe advances on the central role of Fd and its evolutionary role from cyanobacteria to algae/plants. We compare structural diversity of Fd partners to understand this orchestrating role and shed light on how Fd dynamically partitions between competing partner proteins to enable the optimum transfer of PSI-derived electrons to support cell growth and metabolism.

Additional key words: cellular metabolism; electron transfer; ferredoxin; global interaction; oxidation-reduction.

Introduction

The discovery of Fd is itself an interesting achievement in the history of biochemistry. Its role in the cellular oxidation-reduction processes is essential in organisms ranging from non-photosynthetic anaerobic bacteria to photosynthetic unicellular and multicellular life forms. It was first discovered and characterized over 50 years ago (Mortenson *et al*. 1962) in an obligate anaerobic non-photosynthetic bacterium, *Clostridium pasteurianum*. It was identified as an iron containing protein that transfers electron from hydrogenases to a variety of acceptors and contains no heme or flavin prosthetic group. Mortenson *et al.* (1962) were the first to call this protein "ferredoxin" (Fd). Dan Arnon and collaborators were the first to investigate the role of Fd in photosynthesis as described over 50 years ago (Tagawa and Arnon 1962). The Arnon lab was key in unifying the observed functions of Fd, previously believed to be executed by several individual proteins: methemoglobin-reducing factor (MRF) (Davenport *et al*. 1952), $NADP^+$ reducing factor (NRF) (Arnon *et al*. 1957) and photosynthetic pyridine nucleotide reductase (PPNR) (Keister *et al*. 1961). These different biochemical processes rely on the donation of an electron from a distinct family of proteins, now accepted to be ferredoxins.

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Abbreviations: APC – allophycocyanin; BR – bilin reductases; CET – cyclic electron transfer; Cyt – cytochrome; Fd – ferredoxin; Fd_{red} – reduced Fd; Fd_{ox} – oxidized Fd; FAD – flavin adenine dinucleotide; FMN – flavin mononucleotide; FNR – Fd-$NADP^+$-reductase; FNR_{red} – reduced FNR; FNR_{ox} – oxidized FNR; FTR – Fd:Tro reductase; GnS – glutamine synthase; GS – glutamate synthase; K_d– dissociation constant; K_{et} – electron transfer rate constant; NDH – NADPH dehydrogenase; NiR – nitrite reductase; NR – nitrate reductase; PC – phycocyanin; PCB – 3E/3Z phycocyanobilin; PE – phycoerythrin; PEB – 3Z/3E phycoerythrobilin; PΦB – 3E/3Z phytochromobilin; RMSD – root-mean-square deviation; SiR – sulfite reductase; Tro – thioredoxin.
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Since the discovery of Fd, its role has been extensively studied and identified to function in several processes across almost all living organisms. In oxygenic phototrophs, Fd plays a vital role in the electron transport chain as the ultimate electron acceptor from PSI, although in some cyanobacteria and algae, flavodoxin may be expressed and provide the same role under iron-limiting conditions where Fd is not expressed (Sétif 2001). When illuminated, electrons from PSI primarily follow a linear path to reduce Fd and subsequent downstream targets such as Fd-$NADP^+$ reductase (FNR), which catalyzes the reduction of $NADP^+$ to NADPH. NADPH serves as the electron donor for the reduction of 1,3-bisphosphoglyceric acid to glyceraldehyde-3-phosphate, driving an important step in carbon dioxide assimilation in all plants and photosynthetic microbes (C_3, C_4, CAM, algae and cyanobacteria). But the electron donation function of Fd is not limited to FNR, since it is now clear that Fd also serves as a major electron donor to many other partners including, but not limited to: proton reduction by [2Fe-2S]-hydrogenases, sulfite to sulfide reduction catalyzed by an Fd-dependent sulfite reductase, reduction of nitrite to ammonia by Fd-dependent nitrite reductase, cyclic electron transport for generation of a steeper proton gradient necessary for ATP production, reduction of thioredoxins that are involved in the regulation of carbon assimilation and phytobilin synthesis mediated by reduction of biliverdin, catalyzed by Fd-dependent bilin reductases (Beale and Cornejo 1991, Luque *et al*. 1994, Staples *et al*. 1996, García-Sánchez *et al*. 1997, Brand *et al*. 1989). Fds act as an electron carrier that shuttles electrons to diverse redox driven metabolic pathways. In most plants and photosynthetic cyanobacteria, Fds are known to have [2Fe-2S] cluster coordinated by protein cysteine residues which act as the central acceptor site for the transfer of electrons from PSI. Fd carries a single electron. The redox state of both Fe in the oxidized protein is 3^+ and when reduced only one of the Fe becomes 2^+. In algae and higher plants, the redox potential of Fds differs due to varying isoforms, which are grouped phylogenetically into source tissues (phototrophic) and sink tissue such as roots (Gou *et al*. 2006). The crystal structure of the first plant-type Fd was obtained from the cyanobacterium *Spirulina platensis* with a resolution of 2.5 Å (Tsukihira *et al.* 1981, Bes *et al*. 1999). Fd shares a compact structural fold (also known as the Fd-fold) with several metal-binding proteins which is composed of 2 α helices and 4 β strands (Matsuoka and Kikuchi 2014). The overall structure of Fds in cyanobacteria, algae and higher plants varies with a mass range of 10–30 kDa but all retain a conserved amino acid motif ($CX_4CX_2CX_nC$) for the proper assembly of the [2Fe-2S] cluster that leads to the formation of mature protein from apoprotein (Kameda *et al*. 2011). This conserved sequence can be observed in the alignment of the region generated in *Clustal Omega* (Sievers *et al*. 2011) shown in Fig. 1.

The central role of Fd to many cellular processes is intriguing due to its relatively conserved structure. It is therefore interesting how it can function with so many partner proteins. Our aim is to understand how this is possible. More specifically, our interest is to look into the need for this conserved nature of structure and the coordination of its 2Fe-2S center close to the surface of electron transfer and the need to allow docking of Fd at the electron donor site. Fig. 2*A* shows a superimposition of six different Fds generated using *MOE*^{TM} (Molecular Operating Environment 2017) and Fig. 2*B* shows the RMSD value generated in *R* with "corrplot" package and *RColorBrewer* (R Core Team 2011, Wei 2017, Neuwirth 2014). It is clear from this figure that from primitive cyanobacteria to higher plants, the overall structure of Fd remains highly conserved. Investigation of the presence of structural variation and overall flexibility might allow the interaction of Fd with all its electron transfer partners.

The major goal of this review is to delineate the role of Fd as a central hub that connects the photosynthetic electron transport system to the larger network of overall cellular metabolism. We present recent findings throughout the past decade to highlight the integral importance and evolutionary significance of Fds from photosynthetic cyanobacteria to algae and higher plants. We compare the expression and structural diversity of different Fd gene products to aid in understanding their roles in these organisms.

Ferredoxin: NADPH formation

One major role of Fd that has been extensively studied is the reduction of FNR to generate NADPH. Fd accepts an electron from PSI and two of these reduced Fds diffuse in the stromal side of the thylakoid membrane. These two electrons then reduce one $NADP^+$ and an H^+ to form NADPH catalyzed by FNR. FNR has a single non-covalently bound prosthetic group called flavin adenine dinucleotide (FAD), which gets reduced by an electron donated by the first Fd to form a semiquinone form of FAD, followed by a completely reduced form facilitated by an electron donated from the second Fd (Kovalenko *et al*. 2010). This event can be summarized in the following equations:

$$2\ Fd_{red} + NADP^+ + H^+ \rightarrow 2\ Fd_{ox} + NADPH$$

$$Fd_{red} + FNR_{ox} \underset{K_d}{\leftrightarrow} [Fd_{red}\text{---}FNR_{ox}] \xrightarrow{K_{et}} Fd_{ox} + FNR_{red}$$

In the above equations, K_d is the dissociation constant for the formation of the singly reduced intermediate complex, while K_{et} is the electron transfer rate constant. The first X-ray crystal structure of the Fd-FNR complex was solved in *Anabaena* PCC 7119 at a resolution of 2.4 Å

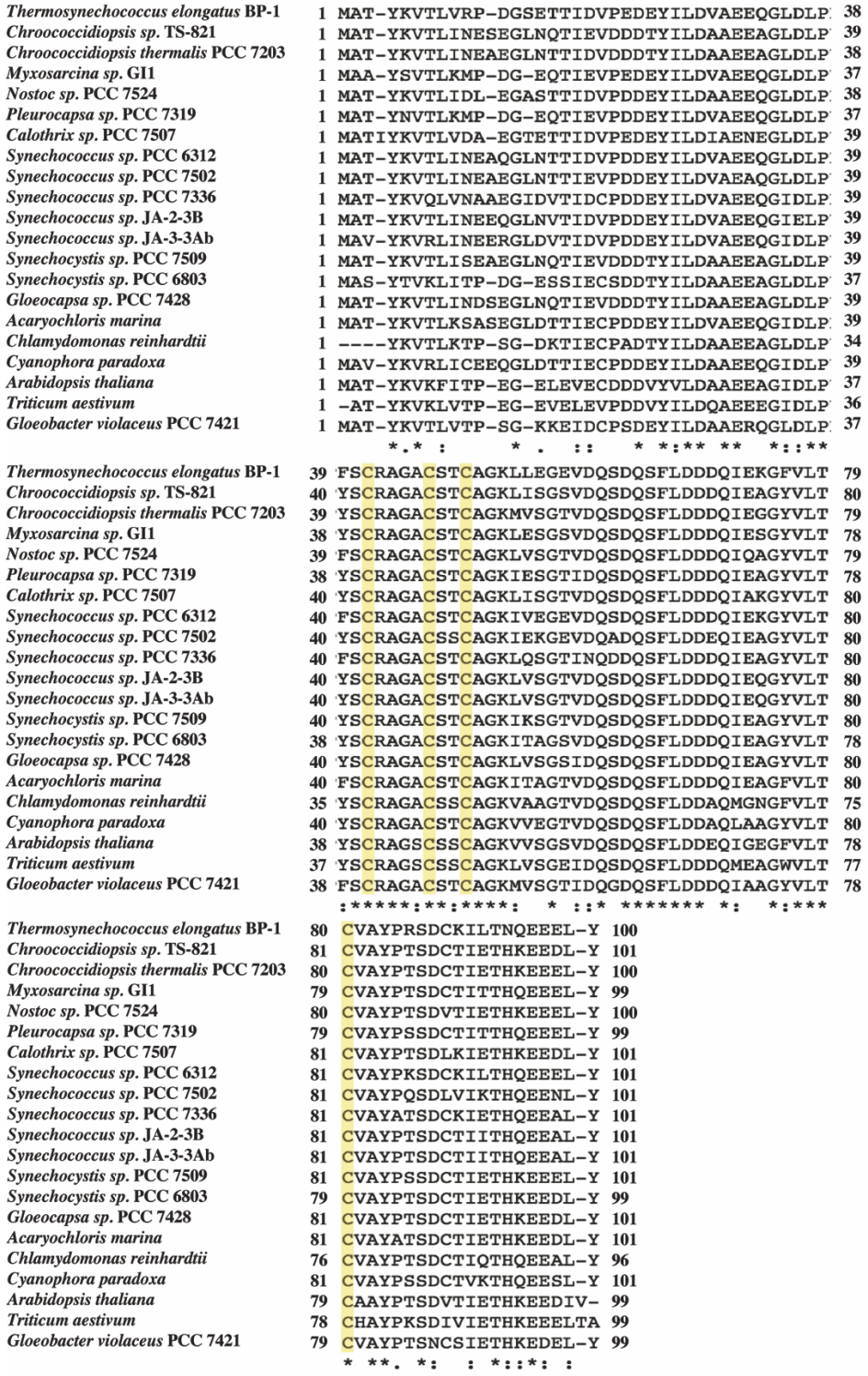

Fig. 1. Conserved amino acid motif of Fd for [2Fe-2S] cluster assembly. Multiple amino acid sequence alignment of the major plant type Fd (PetF) found in cyanobacteria (*Thermosynechococcus elongatus* BP-1, *Chroococcidiopsis* sp. TS-821, *Chroococcidiopsis thermalis* PCC 7203, *Myxosarcina* sp. GI1, *Nostoc* sp. PCC 7524, *Pleurocapsa* sp. PCC 7319, *Calothrix* sp. PCC 7507, *Synechococcus* sp. PCC 6312, *Synechococcus* sp. PCC 7502, *Synechococcus* sp. PCC 7336, *Synechococcus* sp. JA-2-3B, *Synechococcus* sp. JA-3-3Ab, *Synechocystis* sp. PCC 7509, *Synechocystis* sp. PCC 6803, *Gloeocapsa* sp. PCC 7428, *Acaryochloris marina*, *Gloeobacter violaceus* PCC 7421), a glaucophyte (*Cyanophora paradoxa*), a single-cell eukaryotic algae (*Chlamydomonas reinhardtii*) and plants (*Arabidopsis thaliana* and *Triticum aestivum*) constructed by *Clustal Omega* program (Sievers *et al.* 2011). The conserved cysteine motifs (CX4CX2CXnC) are highlighted in yellow boxes.

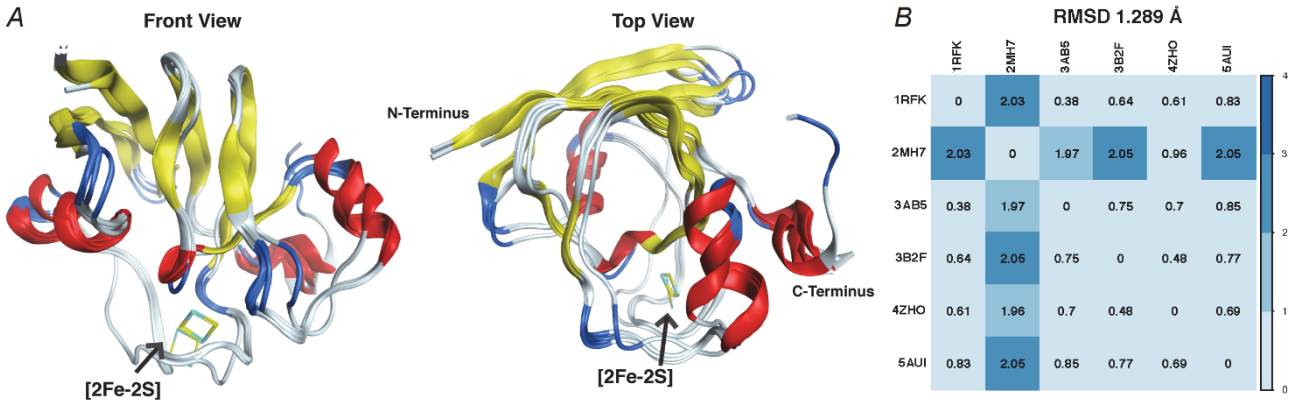

Fig. 2. Conserved tertiary structure of PetF. (*A*) Superimposition of 6 different Fds with PDB IDs-1RFK (*Mastigocladus laminosus*), 2MH7 (*Chlamydomonas reinhardtii*), 3AB5 (*Cyanidioschyzon merolae*), 3B2F *(Zea mays*), 4ZHO (*Arabidopsis thaliana*), and 5AUI (*Thermosynechococcus elongatus* BP-1). The protein sequences were aligned and superimposed using the program *MOE*^{TM} (Molecular Operating Environment 2017). The [2Fe-2S] cluster, in yellow and blue sticks, is indicated by the arrow head in both front (left) and top view (right) and the N- and C-terminal ends are shown in the top view. (*B*) This shows the RMSD calculation of the generated superimposed structure with an average value of 1.289 Å.

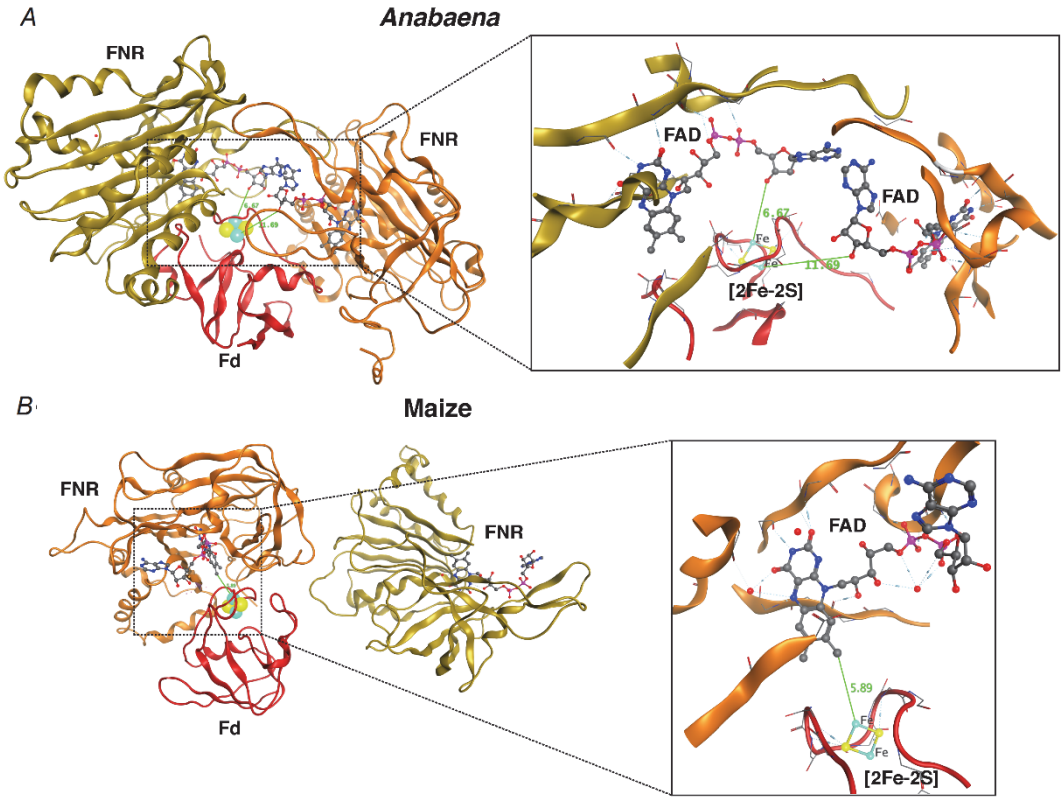

Fig. 3. Crystal structure of Fd-FNR complex. (*A*) The crystal structure of the Fd-FNR complex (PDB ID-1EWY) in *Anabaena* PCC 7119 is shown here derived from Morales *et al.* (2000). The FNR is shown here as a dimer (gold and orange) and the Fd (red). The distance between [2Fe-2S] cluster (yellow and light blue) of Fd and FADs (ball and socket) of the FNR dimer was calculated in Å in *MOE*^{TM} as indicated by green lines. The interaction is symmetrical suggesting that the electron can be accepted by either of the FADs. The amino acid backbone associated with the [2Fe-2S] and FAD interaction are indicated in the zoomed box. (*B*) The crystal structure of the Fd-FNR complex (PDB ID-1GAQ) in maize is shown here derived from Kurisu *et al.* (2001b). The FNR is shown here as a heterodimer (gold and orange) and the Fd (red). Here, the Fd preferentially interacts with one of the FNR chain (Chain A, orange).

(Morales *et al*. 2000) (Fig. 3*A*; derived from Morales *et al*. 2000). This crystal structure (1EWY) of the complex indicates many electrostatic interactions between the two proteins. In higher plants, the three-dimensional structure

of this complex has been determined following isolation from maize leaf at a resolution of 2.59 Å shown in Fig. 3*B* (Kurisu *et al*. 2001a,b). Analysis of these two structures reveals that the distance between the redox centers, namely the [2Fe–2S] cluster of Fd and FAD of FNR are located at a distance of 5.89–6.67 Å apart, and illustrate several intermolecular interactions that mainly include salt bridge formation and hydrophobic interactions at the interface near these prosthetic groups (Kurisu *et al*. 2001a). The complex leads to formation of additional hydrogen bonds between the interacting surface side chains of FNR, indicating structural changes in both FNR and Fd that strengthen the interaction and optimize the orientation of the two proteins to permit rapid electron transfer. Fig. 4*A* highlights the residues that are involved in Fd-FNR complex formation analyzed from *CoCoMaps* (*bioCOmplexes COntact MAPS*) tool (Vangone *et al.* 2011, 2012). Table 1 shows detailed information concerning accessible surface area (ASA), measured in Å^2, of the residues at the interfaces (defined by the resulting buried protein surface due to complex formation).

Multiple studies utilizing site-specific mutagenesis, transient kinetics and stopped-flow assays have been performed to understand the FD/FNR interaction in cyanobacteria and higher plants (Hurley *et al*. 1999, 2006). Through the use of site-specific mutants, these works have shown that specific positively charged amino acid residues on the surface of FNR are important for the binding of Fd. Brownian dynamics (BD) has been employed to investigate the formation of the FD-FNR complex to understand the kinetic parameters for this protein–protein interaction (Kovalenko *et al*. 2010, 2011). These studies reveal rate constants for complex formation between wild type and mutant FNRs which demonstrates a non-monotonic dependence of the binding rate constant on the ionic strength. They also provide insights on the importance and specificity of several electrostatic interactions. It may be noted that in most cyanobacteria and algae under low-iron

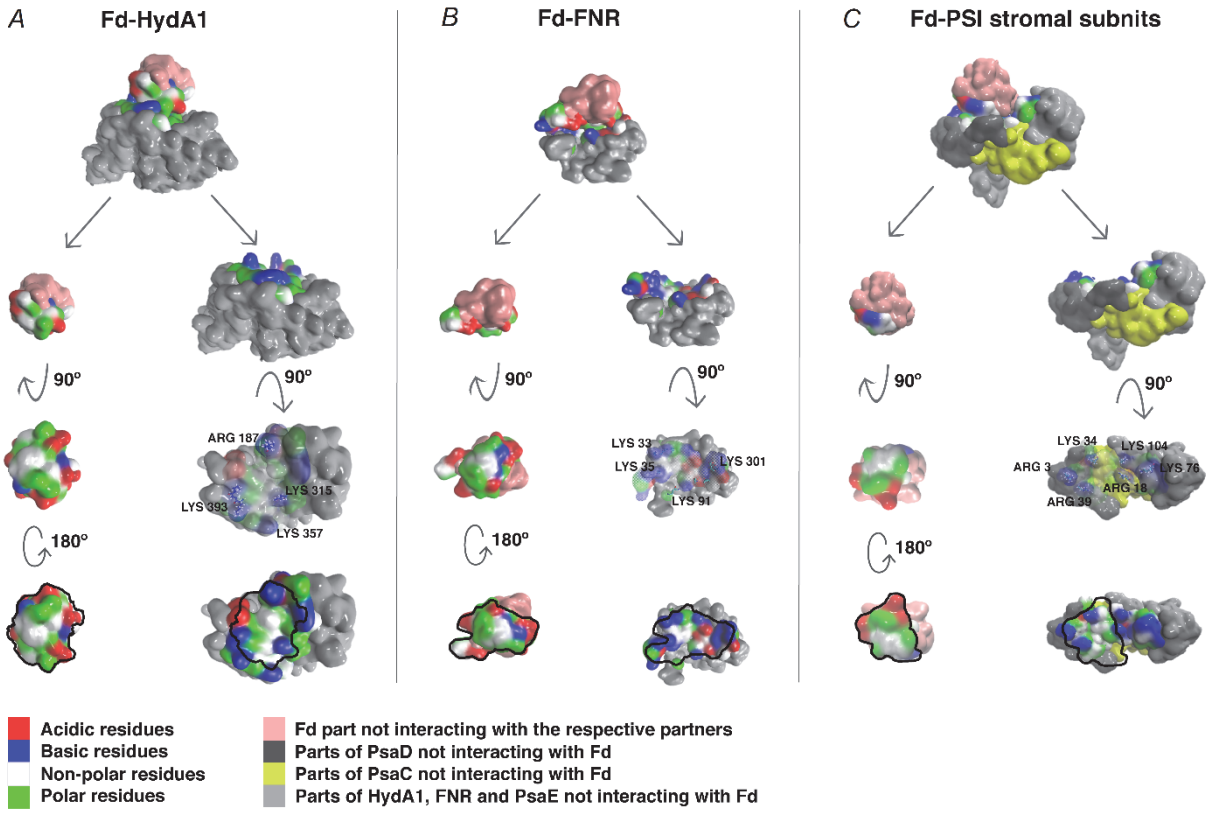

Fig. 4. *CoCoMaps* interaction of Fd-complexes. The crystal structures of (*A*) Fd-HydA1 complex from *Chlamydomonas reinharditii* (PDB ID: 2N0S) and (*B*) Fd-FNR from maize (PDB ID: 1GAQ); and (*C*) docked model structure of Fd-PSI stromal subunits (PsaC, PsaD, and PsaE) from *Thermosynechococcus elongatus* BP-1 were analyzed in the *CoCoMaps* server for investigating the interaction maps of the binding interface of the respective Fd complexes. The final models generated from *CoCoMaps* server were viewed in *VMD* software and the structures were generated in surface-view format. In each panels (*A*–*C*), the sequence of structures shown includes (from top to bottom) – (very top) the overall complex; followed by Fd and the respecting binding partner protein in isolation; 90° rotation of the isolated partners to depict the interacting faces including highlighted residues in HydA1 (ARG 187, LYS 315, LYS 357 and LYS 393), FNR (LYS 33, LYS 35, LYS 91 and LYS 301) and PSI stromal subunits (ARG 3, ARG 18, LYS 34, ARG 39, LYS 76 and LYS 104); and (very bottom) 180° rotation of respective Fds in each complexes to show the region where Fd interacts (bordered in black).

Table 1. Accessible surface area (ASA) details from *CoCoMaps* tool: This section includes a list of Fds and the residues of the respective partner proteins (HydA1, FNR and PSI stromal subunits - PsaC, PsaD, and PsaE) at the interface, defined as those having an ASA decreased by > 1.0 Å upon the complex formation. ASA values in the complex and in the isolated molecule ("free"), and the difference between them are reported for each residue. The percentage of buried surface upon complex formation is also reported.

Property	HydA1	FNR	PsaC	PsaD	PsaE	PsaC + PsaD + PsaE
Buried area upon the complex formation [Å^2]	1,866.1	1,596	451.8	326.1	380.8	1158.7
Buried area upon the complex formation [%]	8.25	8.17	4.22	2.23	3.8	10.25
Interface area [Å^2]	933.05	798	225.9	163.05	190.4	579.35
Polar buried area upon the complex formation [Å^2]	991.3	810.1	323.3	154.2	178.2	655.7
Polar buried area upon the complex formation [%]	53.12	50.76	71.56	47.29	46.8	165.65
Polar interface area [Å^2]	495.65	405.05	161.65	77.1	89.1	327.85
Non-polar buried area upon the complex formation [Å^2]	875	786.1	128.5	171.9	202.7	503.1
Non-polar buried area upon the complex formation [%]	46.89	49.25	28.44	52.71	53.23	134.38
Non-polar interface area [Å^2]	437.5	393.05	64.25	85.95	101.35	251.55
Residues at the interface (Total)	55	47	20	11	10	41
Residues at the interface of Fd	30	25	11	5	5	21
Residues at the interface of the partner protein	25	22	9	6	5	20

conditions, flavodoxin can replace Fd to interact with FNR to facilitate electron transfer (Goñi *et al*. 2008).

It is known that there are minor structural variations between Fd and flavodoxin structures. They are different in sizes (~11 kDa and 18–20 kDa, respectively), but both protein types are strongly acidic, whereas the PSI stromal surface is mostly positively charged. Therefore, electrostatic forces are of major importance for the interactions between PSI and Fd (or flavodoxin) (Sétif 2001). This raises another question, not discussed in this review, as how flavodoxin takes over Fd under such stress condition to perform the same function.

In plants, such as wheat, maize, rice, and *Arabidopsis*, it has been found that there are four known isoforms of FNR that interact with Fds with varying phosphorylation responses, but the physiological significance of this occurrence is still under investigation (Moolna and Bowsher 2010). These putative phosphorylation sites include serine (SER 75) and threonine residues (THR 104 and THR 293). Phosphorylated FNRs are suggested to play a key role in interaction with Fds, though the dynamics of this interaction are not fully understood and similar scenario has not been addressed in cyanobacteria or green algae.

Ferredoxin and hydrogenase interactions

The direct interaction of Fd with hydrogenases was first discovered in the non-photosynthetic bacterium *Clostridium kluyveri*, over 50 years ago. This work utilized an *in vitro,* hydrogen-linked diphosphopyridine nucleotide reduction assay that confirmed both the involvement of Fd and hydrogenase for hydrogen production (Fredricks and Stadtman 1965). Little attention was given to the mechanism of hydrogen evolution from photosynthetic organisms until 1973, when Martin Kamen's group demonstrated the production of molecular hydrogen from the spinach chloroplast without the addition of external electron donors (Benemann *et al*. 1973). Following this seminal work, many other groups soon utilized similar *in vitro* assays to demonstrate molecular hydrogen creation utilizing bacterial Fd and hydrogenases (Tano and Schrauzer 1975, Fry *et al*. 1977, Chen and Blanchard 1979, R'zaigui *et al*. 1980, Shrestha *et al*. 2000). In photosynthetic organisms, the evolution of molecular hydrogen is restricted to anaerobic or sulfur-deprived conditions. Over the past 15 years, significant progress has been made using green algae as a renewable source of hydrogen production (Melis and Happe 2001). Most of this work has been advanced using a free-living unicellular alga, *Chlamydomonas reinhardtii* (Melis *et al*. 2000, Tsygankov *et al*. 2002, Happe and Kaminski 2002, Forestier *et al*. 2003, Kosourov *et al*. 2003, Ghirardi 2006, Liran *et al*. 2016). In a closed algal bioreactor, sulfur deprivation causes a shift in respiration which then consumes most of the released O_2 yielding a temporal period of anaerobiosis, which increases hydrogenase expression, causing a boost in molecular hydrogen production. These growth conditions overcome the sensitivity of the Fe hydrogenase to O_2 by temporarily separating the process of photosynthetic O_2 evolution and H_2 photoproduction. This method allows a two-stage process of photosynthesis and H_2 production by modulating the availability of sulfur in the media. However, over time the cells will undergo apoptosis, requiring the addition of sulfur and a photosynthetic growth phase. This novel hydrogenase is still dependent on Fd and PSI for the the supply of electrons. According to Appel and Schulz (1998), this mechanism is proposed to function as a photoprotective strategy, where the electron transferred to form H_2 leads to the dissipation of excess reductants under anaerobic conditions. Hydrogen is a relatively benign and membrane permeable gas that can then leave the cell and be captured as a fuel.

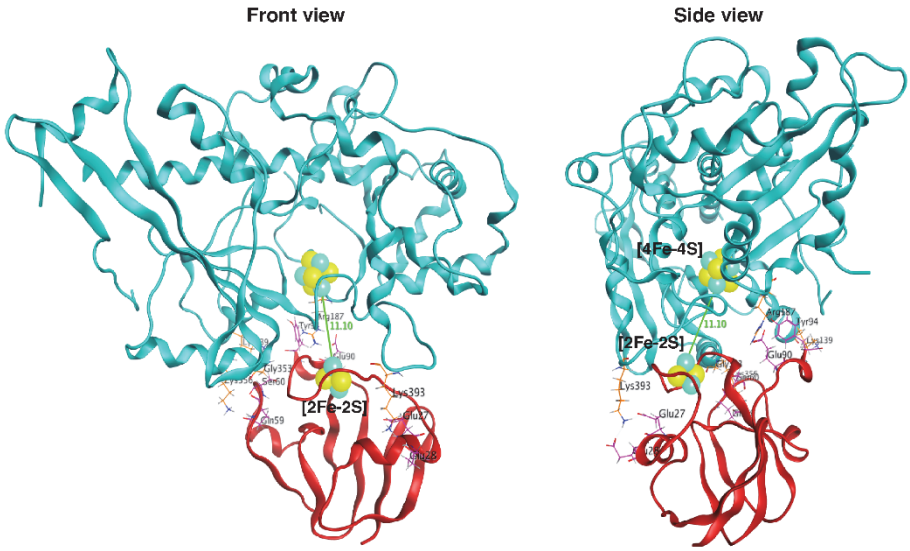

Fig. 5. Crystal structure of Fd-HydA complex. The crystal structure of the Fd-HydA complex (PDB ID-2N0S) in *Chlamydomonas reinhardtii* is derived from Rumpel *et al.* (2015). The distance between [2Fe-2S] cluster of Fd (red) and [4Fe-4S] cluster of HydA in blue (both clusters are shown in yellow and blue balls) was measured to be 11.1 Å in the program *MOE*^{TM}. Some of the hydrophobic and polar residues involved in the complex formation are indicated in both front and side view of the complex.

Two Fe-hydrogenases (HydA1 and HydA2) have been well characterized in *C. reinhardtii*. It is now clear that the transfer of electrons from PSI to the [FeFe] hydrogenase HydA1 in the *C. reinhardtii* requires transfer by PetF (Fd encoded by *petF*). This key step in hydrogen production requires a specific interaction between PetF and HydA1. The transient nature of this electron-transfer complex has thwarted efforts to capture the details of this assembly *via* crystallography. However, despite the elusive nature of this complex, the binding between these hydrogenases with [2Fe-2S]-Fd (PetF) have been shown at atomic resolution by carrying out quantitative binding free energy calculations (Chang *et al*. 2007). According to Chang *et al.* (2007), HydA2 shows a more energetically favored interaction with Fd than HyaA1, with a difference of 83.67 kJ mol^{-1}. One possible model for this interaction is shown in Fig. 5 (derived from Rumpel *et al*. 2015) and a detailed view of the interacting surfaces of both Fd and HydA1 is shown in Fig. 4*B* and Table 1. These authors posit a detailed view of the protein–protein interactions in their model, which include several electrostatic, hydrophobic and hydrogen bonds leading to efficient electron transfer. Interestingly, from the *CoCoMaps* interaction analysis, a common pattern in the interaction face of the electron acceptor proteins – FNR, HydA1, and PSI stromal subunits can be observed (Fig. 4*C* and Table 1; docked model adapted from Cashman *et al*. 2014). To further elucidate the link between all the binding partners of Fd, further investigations on individual docking sites need to be conducted.

Long *et al*. (2008, 2009) utilized Brownian dynamics simulation to show a free-energy landscape of several interacting partners, followed by atomistic molecular dynamic simulations to study their association dynamics. The major conclusion from this study was that the spatial occupancy landscape of the binding partners had a single energy minimum while the orientational occupancy landscapes had multiple minima indicating that the hydrogenase has only one Fd binding site, while Fd itself has multiple binding surfaces that can allow for binding with hydrogenase in multiple favorable orientations. Rumpel *et al.* (2015) applied a similar approach to further investigate the binding sites where they substituted iron with gallium to avoid paramagnetic relaxation enhancement due to Fe. This revealed several hydrophobic and polar residues involved in the formation of the complex. Some of these residues are also shown in Fig. 5.

Cyclic electron transfer

The process of cyclic photophosphorylation has been known for more than 60 years (Arnon *et al*. 1954, Whatley *et al*. 1955). Ten years following this discovery, the involvement of Fd was first proposed by Arnon *et al.* (1967) in spinach chloroplast. Later it was found that cyclic electron transfer (CET) is Fd-dependent, and neither FNR nor cytochrome b_{564} were involved as electron carriers in this pathway (Curtis *et al*. 1973, Böhme 1977, Ivanov and Tikhonova 1979). Though later it was concluded that both Fd and FNR are involved in this pathway, this does not correspond to the NADP$^+$-binding site of the latter (Shahak *et al*. 1981). This indicated that

both CET and non-CET drive photophosphorylation, but the generation of ATP *via* cyclic electron flow is regulated by the level of reduced Fd, and is therefore governed by the NADPH/NADP$^+$ ratio (Hosler and Yocum 1987, Ye and Wang 1997, Krendeleva *et al*. 2001).

Fd transports electrons to the Cyt b_6f complex *via* many pathways (Hanke and Mulo 2013) and continuous CET generates a proton gradient that drives ATP synthesis (Munekage *et al*. 2004). The involvement of two proteins – PGR5 (Munekage *et al*. 2002) and PGRL1 (DalCorso *et al*. 2008) have been shown to act in a regulatory capacity rather than a direct electron mediator from Fd to Cyt b_6f complex in higher plants (Hanke and Mulo 2013, Hertle *et al*. 2013). Many different models have been proposed to describe how Fd facilitates CET (Fig. 6). In algal systems, a supercomplex of PSI, LHCI, LHCII, FNR, Cyt b_6f, and PGRL1 have been discovered in *C. reinhardtii* to control the energy balance (Iwai *et al*. 2010). In cyanobacteria, the involvement of NADPH dehydrogenase (NDH) complex was first demonstrated to undergo CET from Fd to the plastoquinone pool in *Synechosystis* PCC 6803 (Ogawa 1991, Mi *et al*. 1995a,b). It was shown that CET dominated in longer dark exposure due to temporary inactivation of FNR leading to lower linear electron flow (Talts *et al*. 2007). Other stress responses are also proposed to be indicative of CET, for instance drought stress leads to upregulation of PGR5, PGRL1, and FNR while similar to NDH levels and this, in turn, accelerates CET induction (Lehtimäki *et al*. 2010). High heat stress leads to a CET response *via* a NDH-dependent manner in rice to dissipate excess energy otherwise generated by Fd-quinone oxidoreductase-dependent CET (Essemine *et al*. 2016).

Several isoforms of Fd have been identified in plants that are involved in both CET and non-CET, but less is known about the division of labor amongst these isoforms. For instance, in *Arabidopsis*, knockout of the major plant type Fd isoform (Fd2) led to the enhanced expression of non-photosynthetic Fds (Fd3 and Fdc1), with increased expression of minor isoform – Fd1 (a major player involved in CET) under high-light condition (Voss *et al*. 2011). In another study, a transplastomic *Nicotiana tabacum* plant with overexpressed *Pisum sativum* Fd showed that there is an increased CET response even under optimal greenhouse growth conditions (Blanco *et al*. 2013).

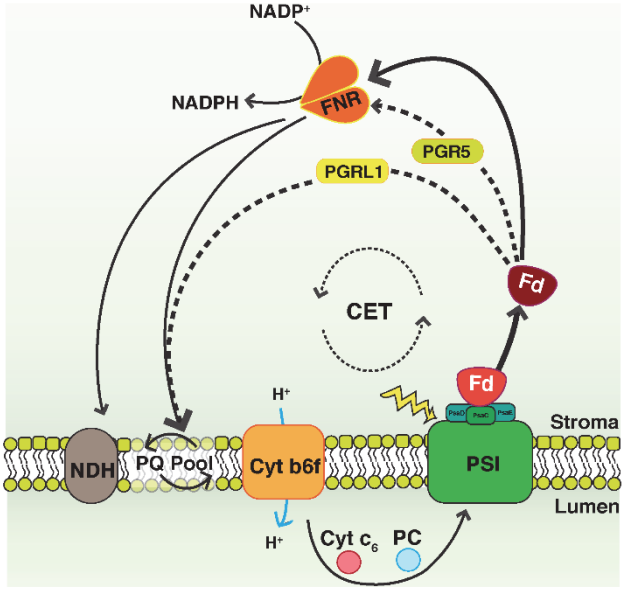

Fig. 6. Schematic of cyclic electron transfer. A schematic representation of the overall pathways of CET is indicated. Upon reduction of Fd (brown) from its oxidized form (bright red) by accepting electron from the stromal subunits (PsaC/D/E) of PSI, the electron is either accepted by FNR directly or PGR5-dependent manner which reduces NADP$^+$ to form NADPH. The flow of electron back to the PQ pool is either mediated my PGR5 or PGRL1 in higher plants or PGRL1 alone (along with FNR in algae) or *via* NDH in cyanobacteria.

Ferredoxin in nitrogen assimilation

For organisms like nitrogen-fixing cyanobacteria and plants associated with such cyanobacteria by symbiosis, this nitrogen assimilation is a major process that requires a significant source of electron donation. It is also clear that even photosynthetic organisms that are non-nitrogen-fixing still require a major electron source to grow under nitrogen limiting conditions. In both cases, there remains the need to facilitate the reduction of nitrate to nitrite by nitrate reductase (NR). NR is a homodimeric protein that contains a molybdenum cofactor, a flavin and a *b*-type cytochrome. NR utilizes two electrons from NADPH to reduce NO_3 to NO_2. This is followed by reduction of nitrite to ammonia catalyzed by nitrite reductase (NiR) (Foyer *et al*. 2001). NiR has two electronically coupled prosthetic groups (one siroheme group and one [4Fe-4S] cluster) and the latter is involved in accepting the electron from the [2Fe-2S] cluster of Fd (Swamy *et al*. 2005) and requires 6 electrons from Fd to drive the reduction reaction (Hase *et al*. 2006). Glutamine synthase (GnS) catalyzes the conversion of glutamate to glutamine using the ammonia, and glutamine along with 2-oxoglutarate forms two molecules of glutamate reduced by glutamate synthase (GS) (requires 2 electrons) (Suzuki and Knaff 2005). In cyanobacteria, NR, NiR (Manzano *et al*. 1976) and GS all require Fd as the electron donor. However, in algae and higher plants, Fd is only associated with NiR and GS only, with NiR utilizing NADPH as the electron donor (Hase *et al*. 2006). Major studies on the binding interaction of Fd with all three enzymes involved in nitrogen assimilation was carried out in both cyanobacteria and higher plants for almost two decades starting in the early 1990s (Manzano *et al*. 1976, Schmitz and Böhme 1995, Gutekunst *et al*. 2014, Srivastava *et al*. 2015). These three reactions are shown in Fig. 7.

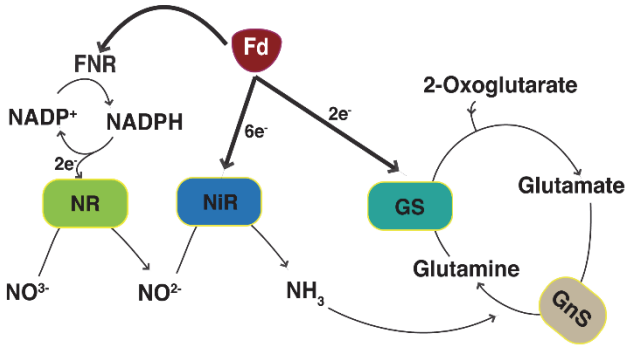

Fig. 7. Schematic of nitrogen assimilation mediated by Fd. A schematic overview of the three major reactions involved in Fd-mediated nitrogen assimilation is shown here. NR (light green) takes 2 e^- from NADPH (which is obtained by FNR upon reduction by Fd) to reduce NO^{3-} to NO^{2-}. This is followed by reduction of NO^{2-} to NH_3 catalyzed by NiR which requires 6 e^- from Fd to catalyze this reaction. GnS catalyzes the conversion of glutamate to glutamine using the NH_3 where glutamine along with 2-oxoglutarate forms two molecules of glutamate reduced by GS using 2 e^- from Fd.

The involvement of aromatic residues of Fd (Phe and Trp) was shown in the case of NRs and NiRs, respectively (Schmitz and Böhme 1995, Tripathy *et al*. 2007). Very little is known about the Fd-NiR complex formation and the involvement of the two prosthetic groups (Mo cofactor and [4Fe-4S]) of the latter (Srivastava *et al*. 2015). Recently, an *in-silico* model of the Fd-dependent NiR from *Synechococcus* sp. PCC 7942 and site-directed mutagenesis studies revealed amino acid residues that may play a major role in either complex formation, prosthetic group binding or catalysis (Srivastava *et al*. 2015). Detailed characterization of Fd-dependent NiR had been conducted by several groups (Privalle *et al*. 1985, Mikami and Ida 1989, Arizmendi and Serra 1990, Hirasawa *et al*. 1994, 2009, 2010; Dose *et al*. 1997, Yoneyama *et al*. 2015).

In 1991, Hirashawa *et al.* (1991) showed the involvement of Fd as an electron donor to glutamate synthase using chemical-crosslinking assay and illustrated the involvement of basic residues (arginine and lysine) in the binding site of Fd at the location similar to its interaction with other binding partners (Hirasawa and Knaff 1993, Hirasawa *et al*. 1993). Glutamate synthase also has two prosthetic groups – one [3Fe-4S] cluster which accepts an electron from Fd and one non-covalently bound flavin mononucleotide (FMN) cofactor (Hirasawa *et al*. 1996). Recent studies reveal that the reduction of this enzyme by Fd is strictly dependent on the presence of NADPH (Yoneyama *et al*. 2015).

Nitrogen being a primary nutrient for plants also becomes the major limiting factor for plant productivity from an agricultural point of view as plants require the help of diazotrophic bacteria to carry out the conversion of atmospheric N_2 to NH_3. The enzyme involved in this process is nitrogenase (Halbleib and Ludden 2000):

$$N_2 + (6 + 2\,n)\,H^+ + (6 + 2\,n)\,e^- \rightarrow 2NH_3 + n\,H_2\ (n \geq 1)$$

The CET module in diazotrophic bacteria involves NifJ (pyruvate oxidoreductase) (Schmitz *et al*. 1993) and NifF (flavodoxin) module which, upon replacement with plant type FNR and Fd (respectively from different plant organelles), showed significant nitrogenase activity, thus suggesting the potential for a FNR-Fd module in biological nitrogen fixation (Tano and Schrauzer 1975, Yang *et al*. 2017).

Sulfite reduction

The reduction of sulfite to hydrogen sulfide was first shown in a thermophilic sulfate reducing non-photosynthetic bacterium, *Clostridium nigrificans* by a dissimilatory pathway (Akagi 1965). In photosynthetic organisms, the assimilation of sulfur involves the ATP-dependent conversion of sulfate to 5'-adenylylsufate, which gets reduced by 2 electrons to form sulfite and AMP. Sulfite reductase (SiR) catalyzes the reduction of sulfite to sulfide using 6 electrons (Setya *et al*. 1996, Nakayama *et al*. 2000). SiR, isolated from *Spinacia oleraea*, was shown to be dependent on Fd as the primary electron donor (Hennies 1975). Plants and cyanobacterial SiR is comprised of one [4Fe-4S] cluster and one siroheme prosthetic group (Krueger and Siegel 1982a,b). Fd-dependent SiR was also isolated and characterized from the red alga *Porphyra yezoensis* (Koguchi and Tamura 1989). It is known that SiR and nitrite reductase have structural and functional resemblance but, interestingly, SiR in the unicellular red alga *Cyanidioschyzon merolae* preferentially reduces nitrite, playing important role in nitrate assimilation (Sekine *et al*. 2009).

As far as Fd interacting with SiR is concerned, it has been shown that the acidic residues of Fd are necessary for the interaction, and the site is partly distinct to that of its interaction with FNR (Akashi *et al*. 1999). On the other hand, SiR has a patch of basic residues in a region distal to the siroheme group that serves as the binding site for reduced Fd (Nakayama *et al*. 2000). Site-specific mutation, chemical shift perturbation and cross saturation experiments conducted by Saitoh *et al.* (2006) confirmed two major acidic patches in Fd, that serve as the SiR binding site, are important for electron transfer as well.

Recently, Kim *et al*. (2016a) revealed in their study that Fd:SiR complex formation and inter-protein affinity are thermodynamically adjusted by both enthalpy and entropy through electrostatic and non-electrostatic interactions, which confirmed that non-covalent inter-protein interactions contribute to maximum enzymatic activity under physiological salt condition. Kim *et al*. (2016b) were also able to co-crystalize the Fd:SiR complex and reveal that multiple conformational states exist for the complex. Though there are differences in the interaction patterns, the

optimum distance for efficient electron transfer between the [2Fe-2S] cluster of Fd and [4Fe-4S] cluster of SiR is maintained in all confirmations, thus demonstrating the flexible nature of Fd as an electron donor in multiple redox metabolisms.

Thioredoxins reduction

One major enzyme in photosynthetic organisms, glucose-6-phosphate dehydrogenase, plays a critical role in carbohydrate degradation and is inhibited by light signals, which involve an interconnected regulatory system of Fd, thioredoxins (Tro) and Fd:Tro reductase (FTR) (Droux *et al*. 1987, Buchanan 1991). Other enzymes that are regulated by this system include fructose-1,6-bisphosphatase in the reductive pentose phosphate pathway, NADP-malate dehydrogenase and Rubisco activase (Dai *et al*. 2004). The light signal is transduced in the form of electrons from PSI to Fd which, in turn, are transferred to FTR (which has a [4Fe-4S] cluster). FTR reduces the disulfide bridges of Tro which leads to the regulation of CO_2 assimilation in the chloroplast and photosynthetic cyanobacteria (Buchanan *et al*. 2002). The FTR is a heterodimeric enzyme with distinct Fd and Tro binding sites (Fig. 7 derived from Dai *et al*. 2000). The [4Fe-4S] cluster is close to one side of the heterodimer, and is accessible to Fd while the disulfide bridge is towards the Tro binding site (Dai *et al*. 2000, 2004).

A recent review by the Bob Buchanan group highlighted that the origin of FTR is rooted to primitive bacteria as well as Archaea (Balsera *et al*. 2013). Since FTR is not universally present in oxygenic photosynthetic organisms, it is replaced by NADPH-thioredoxin reductase, though upon addition of FTRv (a variable subunit of FTR) in oxygenic photosynthetic organisms led to its protection from oxygen (Balsera *et al*. 2013).

Phycobillin reduction

Photosynthetic cyanobacteria, rhodophytes and cryptophytes have light-harvesting pigments, a majority of which are classified as phycobiliproteins or phycobilins. They resemble biliverdin and bilirubin in animals while the phytochromes are most prevalent chromophores in in plants (Beale and Cornejo 1991). It was identified for the first time that the enzyme critical for phycobilin biosynthesis, bilin reductase, is Fd dependent. These phycobilins are precursors for the phycocyanin and phycoerythrin components that form the major light-harvesting antenna complex in cyanobacteria, the phycobilisome (Gómez-Lojero *et al*. 2003).

The biosynthetic pathway involves the cleavage of a heme molecule by heme oxygenase to produce biliverdin which is then reduced to form bilirubin by biliverdin reductase (NADPH-dependent) or phycobilins by a plethora of Fd- dependent bilin reductases (BR). Three types of phycobilins are formed (Dammeyer and Frankenberg-Dinkel 2008, Busch *et al*. 2011):

3E/3Z phytochromobilin or PΦB catalyzed by HY2 (PΦB synthase or phytochromobilin: Fd oxidoreductase) which requires 2 e^- from Fd in most land plants and mosses;

3Z/3E phycoerythrobilin or PEB either by (*1*) two-step reaction in red algae and cryptophytes catalyzed by PebA (15,16-dihydrobiliverdin:Fd oxidoreductase) and PebB (phycoerythrobilin:Fd oxidoreductase) (each step requires 2 e^- from Fd); or by (*2*) a single reaction catalyzed by PebS (phycoerythrobilin synthase) in cyanophage infected cyanobacteria (requires 4 e^- from Fd);

3E/3Z phycocyanobilin or PCB catalyzed by PcyA (PCB:ferredoxin oxidoreductase) in most cyanobacteria (Busch *et al*. 2011).

Future directions

Ferredoxin, being a highly versatile electron donor, it is capable of interacting with a host of acceptor proteins (as illustrated in Fig. 8). Although recent work has begun to explore the structural basis for these interactions (Kapoor *et al*. 2018, Marco *et al*. 2018), there is much less work on the regulation of this interaction. In this review, we have tried to explore the multitude of partners with which there are established interactions in phototrophic organisms. Certainly, in the future the number of partners will expand. Current future directions will involve the role of cellular pH and ionic strength (Diakonova *et al.* 2016), transcription control (Domínguez-Martín *et al*. 2017), translational control (Omairi-Nasser *et al*. 2014), post-translational modification (Lehtimäki *et al.* 2014), sub-cellular compartmentalization (Yang *et al.* 2016), supramolecular organization (Kimata-Ariga and Hase 2014), maturation (Van Hoewyk *et al.* 2007), scaffolding proteins (Hu *et al*. 2017, Nath *et al*. 2017), and regulated inactivation/degradation (Vuorijoki *et al*. 2017). Future structural analysis using advanced cryo-TEM methods will allow larger and more labile molecular complexes to be studied that may elude traditional crystallography methods. Together these advances will render new insight into the operations of Fd and its central role in mediating the fate of the PSI-derived electrons to multiple competing metabolic processes.

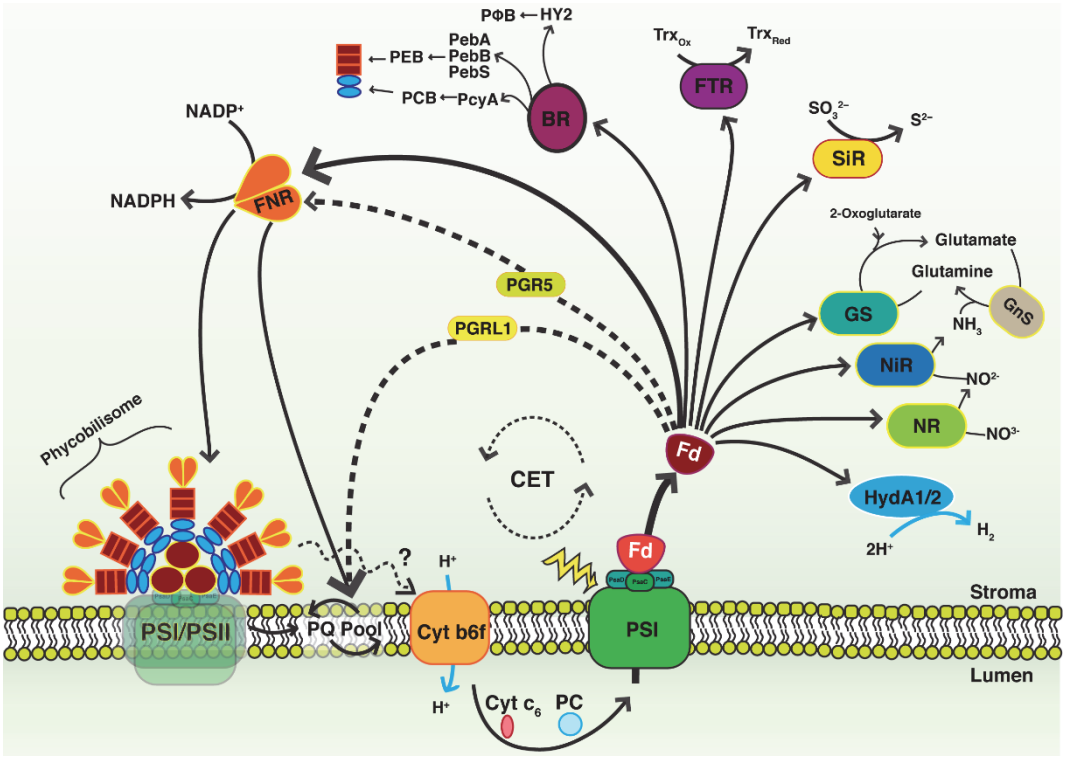

Fig. 8. Global interaction of ferredoxin. Cartoon model showing the global interaction of Fd in cellular metabolism. The "fate of the electron" from reduced Fd (brown) is focused here and it involves a plethora of electron acceptors on the stromal side of the membrane. These include hydrogenase (HydA1/2), NR, NiR, GS, SiR, FTR, BR, and FNR. The alternate route for electron transfer for reduced Fd to FNR is also shown *via* PGR5 and PGRL1 to conduct CET. In cyanobacteria, PEB and PCB become precursors to for phycoerythrin (PE) in and phycocyanin (PC) (red and cyan rectangles, respectively), two major components for the formation of light-harvesting antenna complex called the phycobilisome. It has a allophycocyanin (APC) core (red oval) attached to the PE-PC antenna rods. The phycobilisome complex is located on the stromal side of PSI/PSII. The FNRs are covalently linked with the PE components and *via* this mechanism, they can potentially play a role in trafficking electron to the PQ pool.

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