

A comprehensive study on ferredoxin isoforms in the cyanobacterium *Synechocystis* sp. PCC 6803

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Ferredoxins are central to cellular metabolism by mediating electron flow in energy conversion reactions. The focus of this study was to systematically examine twelve ferredoxin and ferredoxin-like proteins from *Synechocystis* sp. PCC 6803 to identify their properties, activities, and functions in electron transfer. Using electron paramagnetic resonance spectroscopy, we detected cluster types consistent with major ferredoxin families including plant-type [2Fe-2S], adrenodoxin, thioredoxin, and bacterial-type [4Fe-4S] ferredoxins. In addition, we found that the *ssr3184* ferredoxin-like protein exchanged between a [3Fe-4S] or a [4Fe-4S] cluster, pointing to a possible functional change in response to changes in oxygen or cellular redox poise. Electrochemical measurements demonstrated that these ferredoxins constitute a broad potential window, from -243 mV to -520 mV vs SHE. Investigations on their capacity to support electron transfer focused on reactions with two major redox hubs: Photosystem I and pyruvate:ferredoxin oxidoreductase and included testing of binding interactions with nitrite reductase. Expression profiling under multiple environmental conditions was also used to predict function and revealed distinct regulatory patterns. Collectively, these findings identified a group of core ferredoxins that directly support photosynthetic electron transfer, and more specialized ones that may serve other functions. In summary, *Synechocystis* utilizes a suite of ferredoxins to maintain cellular redox homeostasis under dynamic environmental conditions.

Introduction

Every living cell depends on a finely regulated distribution of electrons between a multitude of enzymatic reactions. This complex distribution is the basis for a functioning metabolism and redox homeostasis and is required to adapt to changes in environmental conditions. Ferredoxins (Fdx) play a central role in these

distribution and adaptation processes. They are small redox proteins with [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters, in which the irons exist in different oxidation states to shuttle electrons. Fdxs are ubiquitous in all domains of life and among the oldest proteins that emerged during the origin of life on Earth [1]. [4Fe-4S]

Abbreviations

Fdx, Ferredoxins; Fe-S, iron–sulfur; HoxEFUYH, NiFe hydrogenase; PFOR, pyruvate:ferredoxin oxidoreductase; PSI, photosystem I.

clusters evolved in an anaerobic environment. The emergence of oxygen-tolerant [2Fe-2S] cluster Fdxs marked a major evolutionary turning point, coinciding with the advent of oxygenic photosynthesis [2,3].

Despite their small size, Fdxs differ in cluster composition, redox potential, and interaction partners, allowing them to participate in a wide range of electron transfer reactions [3–6]. In photosynthetic organisms, Fdxs link the photosynthetic electron transport chain to various downstream enzymes involved in cellular metabolism and adaptation to environmental conditions. Fdx diversity reflects an evolutionary specialization in which individual proteins evolved to support diverse reactions, suggesting there has been an evolutionary pressure to select properties for fine-tuning redox flow within specific metabolic contexts and more than simply serving as interchangeable electron carriers.

The model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) encodes a diverse set of iron–sulfur (Fe-S) proteins (Gene ID, Table 1), some with confirmed and others with putative Fdx or Fdx-like functions (for simplicity, we will refer to all as “Fdxs” throughout text). Fdxs 1–4 have been classified as plant-type Fdxs that typically function in photosynthetic electron transfer. Fdx1–Fdx4 have redox midpoint values (E_m) that range from -243 to -460 mV vs SHE [3,5], suggesting functional diversity. Fdx1, is the most studied Fdx and has the primary function in photosynthetic electron transfer from PSI

to Fdx–NADP⁺ reductase (FNR) facilitating NADPH use in the Calvin–Benson–Bassham (CBB) cycle [6,10]. In *Synechocystis* Fdx1 is critical for viability also in the presence of added glucose [10]. Fdx1 also functions in electron transfer to nitrite or nitrate reductases [11], sulfite reductase [12], Fdx-dependent GOGAT [13,14], [NiFe]-hydrogenase complexes HoxEFUYH [15] and HoxEFU [16,17]. In addition to NAD(P)⁺, [18] it may donate electrons to flavodiiron proteins Flv1 and Flv3 [19,20]. Fdx also transfers electrons to phycocyanobilin:ferredoxin oxidoreductase (PcyA) for phycobilisome synthesis [21–24] and is involved in the MEP pathway for isoprenoid synthesis [25,26]. Fdx2, is an essential Fdx with a high (positive) redox potential ($E_m = -243$ mV) [7]. Previous studies suggested that Fdx2 is not involved in photosynthetic electron transfer. Under low-iron conditions, cells with lowered Fdx2 levels fail to accumulate the light-harvesting protein IsiA, suggesting that Fdx2 helps coordinate redox signaling with iron homeostasis [7]. Fdx3 expression is light-dependent with deletion mutants being impaired in photomixotrophic growth, with pyruvate:ferredoxin oxidoreductase (PFOR) as its electron donor [27]. Fdx4 is a low-potential ferredoxin, $E_m = -460$ mV that is induced under CO₂, nitrate, or phosphate limitation [28], as well as oxidative stress. Fdx4 also supports NAD⁺ reduction by HoxEFU [16]. Fdx5 and Fdx6 are [2Fe-2S] proteins with unconfirmed function, while Fdx5 was reported to be essential Fdx6 could be deleted from all genome copies [5]. Fdx5 is

Table 1. Data summary for the 12 Fdx and Fdx-like proteins from *Synechocystis*.

Fdx	Gene ID ^b	Fe-S cluster	Fe-S cluster binding Motif ^b	Classification ^c	E_m (mV vs. SHE) ^d
1	ssl0020	[2Fe-2S]	CX ₄ CX ₂ CX ₂₉ CV	Plant	-412 ± 5 , ref [4] $-418 \pm 12^*$
2	sl1382	[2Fe-2S]	CX ₄ CX ₂ CX ₂₉ CV	Plant	-243 , ref [7]
3	slr1828	[2Fe-2S]	CX ₄ CX ₂ CX ₂₉ CV	Plant	$-464 \pm 11^*$
4	slr0150	[2Fe-2S]	CX ₄ CX ₂ CX ₂₇ CV	Plant	-460 ± 10 , ref [8] $-357 \pm 12^*$
5	slr0148	[2Fe-2S]	CX ₅ CX ₂ CX ₃₅ CQ	Adrenodoxin	-280 , $-430^{*#}$
6	ssl2559	[2Fe-2S]	CX ₄ CX ₂ CX ₃₂ CQ	n.d.	$-421 \pm 112^*$
7	sl10662	[3/4Fe-4S]	CX ₂ CX ₂ CX ₃₆ CP	Bacterial	$-403 \pm 10^*$
8	ssr3184	[3/4Fe-4S] [4Fe-4S]	CX ₅ CX ₃ CPX ₂₅ CX ₂ CX ₂ CX ₃ CP	Bacterial	n.d.
9	slr2059	[4Fe-4S] [4Fe-4S]	CX ₂ CX ₂ CX ₃ CPX ₂₃ CX ₂ CX ₂ CX ₃ CP	Bacterial	$E_{m1} = -420 \pm 9$ $E_{m2} = -516 \pm 10$ ref [9]
10 ^a	sl1584	[2Fe-2S]	CX ₇ CX ₃₉ CX ₃ CQ	Thioredoxin	$-407 \pm 7^*$
11	ssl3044	[2Fe-2S]	CX ₅ CX ₂ CX ₃₂ CQ	Adrenodoxin	-416 , ref [8]
12 ^a	ssr1041	[2Fe-2S]	CX ₄ CX ₂ CX ₁₂ CI	n.d.	n.d.

^aPreviously unreported putative Fe-S binding protein. Fdx12 was not characterized experimentally; cluster type and classification are based solely on the protein sequence; ^bGene ID from KEGG, UniProtKB/Swiss-Prot protein database and Cyanobase, (complete sequences are provided in Fig. S11); ^c*Synechocystis* Fdxs were classified according to characteristic Fe-S binding motifs (26) and structure comparison; ^dFdx midpoint potentials (E_m vs. SHE) were measured by SWV, ^{*} E_m values determined in this work. [#]SWV measurement of Fdx5 gave two different E_m values (Fig. S11). n.d., not determined.

co-transcribed with Fdx4, suggesting coordinated regulation under nutrient limitation, while Fdx6 is upregulated under blue light and phosphate deficiency [29].

Fdx7, Fdx8, and Fdx9 form a distinct subgroup of bacterial Fdx-like proteins containing [4Fe-4S] or [3Fe-4S] clusters. Fdx7 is non-essential under standard growth conditions but becomes critical during oxidative stress, iron limitation, strong light, or exposure to selenate or cadmium [30–33]. Previous studies suggested that Fdx7 and Fdx9 function in a coordinated stress response with the thioredoxin system [5,31]. Fdx8 appears to be essential for growth but has no assigned function [5,29,32]. Based on the protein sequence, it was proposed to bind [3Fe-4S] and [4Fe-4S] clusters. Fdx9 is structurally unique based on a two-domain architecture and harboring two spin-coupled [4Fe-4S] clusters ($E_m = -420$ and -516 mV) [9]. Fdx9 expression is induced under cold, high-light, or oxidative stress conditions [29], and deletion impairs photomixotrophic growth and metal stress tolerance [5]. Fdx9 supports photomixotrophic growth possibly by coupling electron transfer to PFOR [27] and is reported to interact *in vitro* with Flv3 based on two-hybrid assay experiments [5,34].

In this work, we evaluated the structural classifications and properties of the nine known, putative Fdxs and Fdx-like proteins, as well as three newly identified: Fdx10, Fdx11, and Fdx12. We used electron paramagnetic resonance spectroscopy and electrochemistry to refine the assignments of Fe-S cluster type and E_m values, respectively. We examined their expression patterns under a variety of growth conditions and tested reactivity with a few key physiological redox partners, Photosystem I (PSI), pyruvate ferredoxin oxidoreductase (PFOR), and nitrite reductase (Nir), offering new insights into how these proteins contribute to electron circuitry and metabolic regulation [3,5,16,19,35]. We integrate our findings with previously reported analyses to further resolve the roles of Fdx and Fdx-like proteins in the metabolism and biochemical function of electron transfer. Overall, the results advance a deeper understanding of how diversity in the *Synechocystis* Fdx proteins has evolved to support electron flow and maintain cellular redox homeostasis during adaptation to fluctuating environmental conditions.

Results

Sequence and structural comparisons of ferredoxins Fdx1–Fdx12

Fdx1 to Fdx9 were previously described and classified [5]. Three additional Fdxs, Fdx10 (ssl1584) [19], Fdx11

(ssl3044) [16], and Fdx12 (ssr1041) (this study) were identified (Table 1). Fdxs typically coordinate either [2Fe-2S] or [4Fe-4S] cluster(s) [3,35,36]. They are further classified based on amino acid sequence, protein structure, and the cysteine-rich motifs that coordinate the Fe-S cluster(s) (Table 1, Figs 1 and S1) [3,35].

As Fdx1 is the only *Synechocystis* Fdx with experimentally resolved structure [37], protein structures of the other 11 Fdxs were obtained *in silico* (Fig. 1). Similarities between Fdx1 and the other plant-type *Synechocystis* Fdxs were quantified using Root Mean Square Deviation (RMSD) calculations [38]. Calculated RMSD values for Fdx2, Fdx3, Fdx4 and Fdx12 were 0.918, 0.495, 0.457, and 2.806 Å respectively, with values below 2 suggesting significant structural homology. Plant-type [2Fe-2S] Fdxs typically exhibit high sequence and structural homology [3], as holds true for Fdx1, Fdx2, Fdx3, Fdx4, and Fdx12 (Fig. 1A). They adopt a highly conserved β -grasp fold, comprising a mixed β -sheet with five β -strands and one to three adjacent α -helices [3]. The [2Fe-2S] cluster is coordinated by four conserved cysteines in a CX₄CX₂CX_nC motif and further stabilized by a network of hydrogen bonds.

Structures and sequences of Fdx5 and Fdx11 resemble adrenodoxin-type [2Fe-2S] cluster Fdxs (Figs 1B and S2), which are versatile proteins that function as soluble electron carriers in a plethora of reactions. They typically consist of a conserved core domain essential for electron transfer and a variable domain usually including a hydrophobic loop and a flexible C-terminus for the recognition and interaction with their protein partners [39]. Fdx5 is the largest protein in the group, exhibiting the most complex structure, including a flexible domain with a 20 amino acid long α -helix, as well as a less accessible cluster (Figs 1B and S2). Fdx6 seems to have an atypical [2Fe-2S] cluster binding motif, with structural characteristics resembling both plant and adrenodoxin type. Electron paramagnetic resonance (EPR) characterization of this Fe-S cluster is discussed later, but shows features more in line with a plant-type [2Fe-2S] cluster.

Fdx10 is a thioredoxin-like Fdx with a structure that is distinct from other Fdxs and features a highly conserved thioredoxin fold, consisting of a central four-stranded β -sheet surrounded by three α -helices (Fig. 1C) [40]. Notably, Fdx10 contains an atypical Fe-S binding sequence, C₃₂C₃₃X₆C₄₀C₄₁X₃₈C₈₀X₃C₈₄Q, where one cysteine from each Cys-Cys pair, Cys32 and Cys40, coordinates the [2Fe-2S] cluster. The structure suggests that the adjacent cysteines, Cys33 and Cys40, may play a role in stabilizing the cluster or modulating

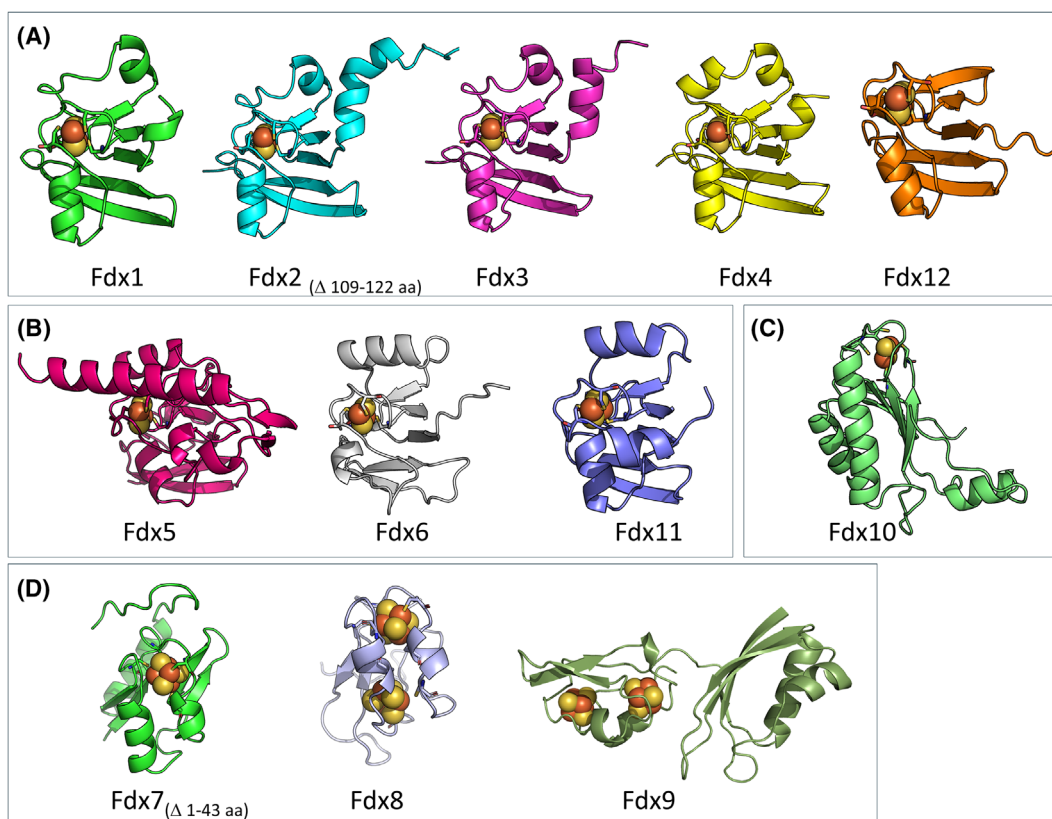


Fig. 1. Comparison of *Synechocystis* Fdx protein structures (generated in Pymol) and classifications (ferredoxins of the same type are boxed). The comparisons reveal the structural variability of *Synechocystis* Fdxs. (A) [2Fe-2S] plant-type; Fdx12 classification was predicted based on the structure and sequence similarity, cluster type and classification were not experimentally confirmed. (B) [2Fe-2S] cluster adrenodoxin-type, Fdx5 and Fdx11; Fdx6, undetermined. (C) [2Fe-2S] thioredoxin-like. (D) [4Fe-4S] bacterial-type. For Fdx1 an experimental structure is available (PDB ID: 1OFF). Structures for [2Fe-2S] cluster Fdxs were predicted using AlphaFold2. The clusters were placed using AlphaFill and minimized using the AMBER forcefield. The Fe-S clusters are shown as spheres while the protein structures are shown as a cartoon representation. Placement of [4Fe-4S] clusters in Fdx7 and Fdx8 were determined based on Pymol generated models using PDBs of structural homologs; PDB ID: 1FXR and PDB ID: 1H98. Truncated versions are shown for Fdx2 ($\Delta 109-122$) and Fdx7 ($\Delta 1-43$), because the respective segments were predicted with low confidence by AlphaFold. Fdx5 was modeled using the short sequence (Fig. S1). The Fdx9 model structure was previously reported [9].

its reactivity, an intriguing possibility that warrants further investigation. The remaining cysteines (Cys17 and Cys64) are far away from the [2Fe-2S] cluster, in positions that could allow for the formation of S-S bonds and a thioredoxin-like function, and may be involved in redox sensing and regulation [41]. The function of adrenodoxin and thioredoxin-type Fdxs remains poorly understood in cyanobacteria, and our analysis provides a first step toward filling this gap by revealing their distinct structural features and potential roles in redox sensing and protein regulation.

The bacterial-type [3Fe-4S]/[4Fe-4S] group includes Fdx7, Fdx8, and Fdx9, each exhibiting $\beta\alpha\beta\beta\alpha$ secondary structure motifs typical of this family. Fdx7, among the largest *Synechocystis* putative Fdxs,

possesses extended N- and C-terminal segments with intrinsic flexibility, possibly mediating complex formation with partner proteins (Table 1, Figs 1 and S3). The position of four of the cysteines suggests they function in binding of a single [4Fe-4S] cluster, while an additional fifth cysteine may contribute to Fe-S cluster stabilization through hydrogen bonding. Based primarily on its amino acid sequence and predicted structural features, Fdx8 is most consistent with the assignment as a [3Fe-4S], [4Fe-4S] di-cluster Fdx (14). Notably, the canonical ligand for a [4Fe-4S] cluster is replaced by a non-polar valine residue, indicating the absence of a fourth, conventional iron-coordinating cysteine. This is supported by sequence alignments with representative $2\times[4Fe-4S]$ or [3Fe-4S], [4Fe-4S]

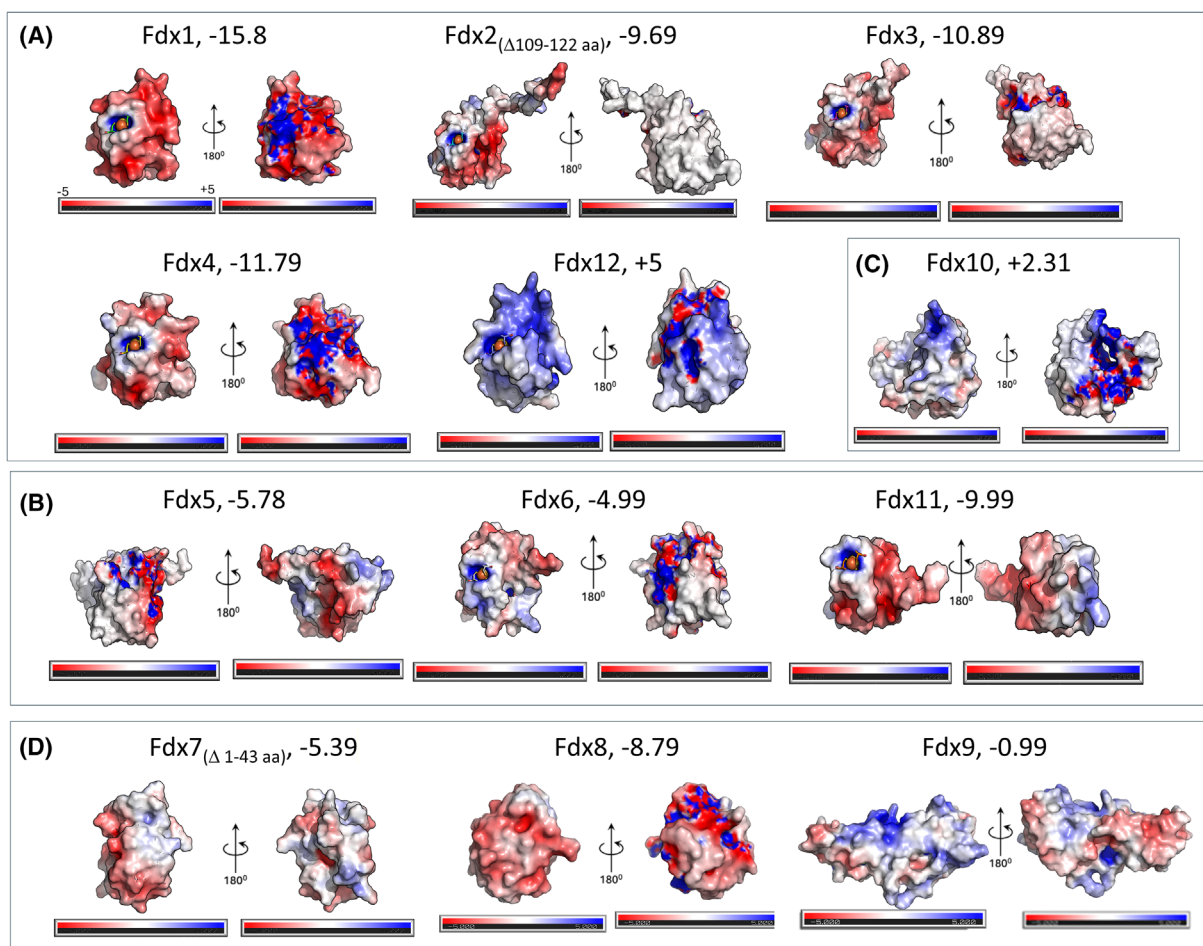


Fig. 2. A comparison of the surface charges of Fdxs 1–12 calculated for pH 7 (ferredoxins of the same type are boxed): (A) [2Fe-2S] plant-type and Fdx12; Fdx12 classification is predicted based solely on the structure and sequence, (B) [2Fe-2S] adrenodoxin-type and Fdx6, (C) [2Fe-2S] thioredoxin-like type, (D) [4Fe-4S] bacteria-type. Surface charges for Fdx9 were reported previously [9]. The surface charge predictions were obtained in Pymol using the APBS surface charge calculation plugin. The blue regions indicate positively charged residues, the red regions indicate negatively charged residues, white regions indicate neutral surface. Due to the regions with poorly defined AlphaFold structure for Fdx2 and Fdx7, calculations may misrepresent the overall surface charges. Fdx5 was modeled using the short sequence (Fig. S1).

di-cluster Fdxs (Fig. S4). A particularly intriguing observation regarding the cluster composition of Fdx8, discussed in more detail later in the text, was an apparent discrepancy between the sequence-based prediction of a [3Fe-4S] and a [4Fe-4S] cluster and the experimentally observed presence of $2 \times [4\text{Fe-4S}]$ clusters. Structural modeling of Fdx8 (Fig. S5) suggests that the amide nitrogen of the valine residue is positioned near the [3Fe-4S] cubane, in a location that could potentially serve in coordinating a fourth iron atom to stabilize a [4Fe-4S] cluster (Fig. S5). However, this hypothesis requires further experimental verification [5]. Fdx9 features an unusual two-domain structure, with one domain resembling a clostridial-type $2 \times [4\text{Fe-4S}]$ Fdx, while the second domain

resembles a Nif-like structure with unidentified function [9].

In silico analysis of Fdx1-12 surface charges

The spatial arrangement of charged amino acids on protein surfaces is known to affect the electrostatic contribution to the formation of protein–protein interactions, which are necessary for electron transfer to a partner enzyme. The strength of the electrostatic contribution directly correlates to the number of complementary surface charges between a protein–protein pair. To assess the distribution of charged residues on the surfaces of Fdx1–Fdx12 (Table S1), we performed an *in silico* analysis on each of the structural models (Fig. 2).

Plant-type Fdxs generally exhibit acidic surfaces that favor interaction with positively charged partners involved in photosynthetic electron transfer and downstream metabolism [36]. Consistent with this, Fdx1 and Fdx4 show highly acidic surfaces, whereas Fdx2 and Fdx3 combine acidic and hydrophobic regions, implying alternative binding partners. Fdx5 and Fdx6 display mixed acidic and basic patches typical for adrenodoxin-type Fdxs [37], supporting versatile electrostatic and hydrophobic interactions. Among the bacterial-type Fdxs, Fdx7 and Fdx8 are overall acidic. In contrast, Fdx10 and Fdx12 show basic surfaces, unusual for proteins with Fdx function [6,9,42].

Comparative biophysical characterization of Fdx1-Fdx11

Synechocystis Fdx1-Fdx11 and IsiB (flavodoxin) were over-expressed in *E. coli* and purified (Table S2 [9,43], Fig. S6), while Fdx12 could not be recovered as a soluble protein from *E. coli* and will have to be the subject of future studies. To assess the purity and integrity of the purified proteins and to further characterize their Fe-S clusters, UV-Vis absorption spectra (Figs S7 and S8) and electron paramagnetic resonance (EPR) spectroscopy spectra (Figs 3 and S9 and Table S3 [9]) were recorded.

A comparative analysis of UV-Vis spectra for Fdx1, Fdx7, and Fdx8 with oxidized [2Fe-2S] and [4Fe-4S] clusters, respectively, illustrates characteristic spectral features of different cluster types (Fig. S8A). All *Synechocystis* [2Fe-2S] cluster Fdxs exhibit similar UV-Vis absorption profiles, with characteristic peaks between 414–426 nm and 451–470 nm (Fig. S7). The primary distinction between spectra from different [2Fe-2S] cluster Fdxs lies in the relative intensities of the peaks at 420 nm and 460 nm. Fdx7 and Fdx8 spectra display broader, less resolved peaks around 420 nm, a feature characteristic of oxidized [4Fe-4S] clusters. The UV-Vis spectra of all reduced Fdxs show lower intensity and broader peaks, consistent with reduced Fe-S clusters with the exception of Fdx5 which displayed a similar level of absorption upon addition of NaDT (Fig. S7). This may indicate an inability of Fdx5 to be reduced with NaDT under the conditions that were applied to other Fdxs though other reducing reagents were not examined in this study. While the UV-Vis data is consistent with the EPR of Fdx5 reduced with NaDT which only displayed a weak signal (vide infra), additional studies are necessary to determine the exact nature of the cluster.

Notably, anaerobically purified Fdx8 exhibits spectral features that are characteristic of [4Fe-4S] clusters (Fig. S8) [44] despite its sequence suggesting a more

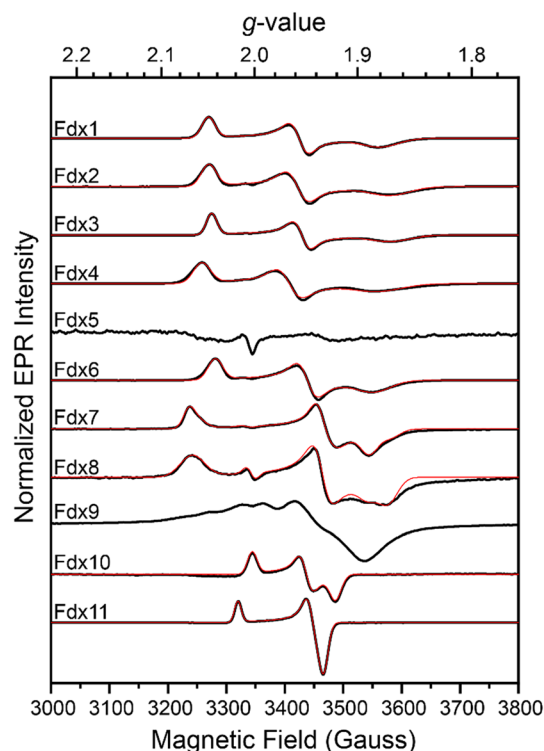


Fig. 3. Electron paramagnetic resonance spectra of reduced Fdx1–11. Fdx samples were reduced with sodium dithionite. Experimental traces ($n = 1$; due to sample scarcity) are shown in black and simulated traces are shown in red. Fdxs 8 and Fdx9 (spectrum previously reported in Ref. [9]) were reconstituted with iron and sulfide under anaerobic conditions. A simulation was not carried out for Fdx5 due to its overall weak nature. A simulation for Fdx9 was omitted due to spectral complexity from spin–spin coupling [9]. All spectra were recorded at 1 mW microwave power. Fdx1 to Fdx8 were recorded at 20 K, Fdx9 at 10 K [9], Fdx10 and Fdx11 at 30 K.

likely [3Fe-4S] [4Fe-4S] configuration (Figs S4 and S5). Upon exposure to air, broad peaks appear around 315 nm and 410 nm, indicative of the formation of oxidized [3Fe-4S] clusters [44]. These experimental results suggest that one of the Fdx8 clusters, upon exposure to oxygen, transforms from a [4Fe-4S] to a [3Fe-4S] cluster (Fig. S8) [9]. The physiological significance of an interchangeable [3/4Fe-4S] cluster remains to be investigated.

Electron paramagnetic resonance spectroscopy

A survey of the EPR spectral properties of reduced Fdxs from *Synechocystis* was carried out to aid in the assignment of the Fe-S cluster type, and to inform on the various cluster geometric and magnetic properties (Figs. 3 and S9, Table S3 [4,9,45,46]).

Reduced Fdx1–4 exhibit similar spectra represented by rhombic $S = \frac{1}{2}$ signals with calculated rhombicity values typical for plant-type [2Fe-2S] cluster Fdxs (Table S3) [4,47,48]. Slight variation in the degree of rhombicity is indicative of small differences in the distortion of the Fe^{2+} environment for the mixed-valent ($\text{Fe}^{2+}\text{-Fe}^{3+}$) [2Fe-2S] cluster [45]. Fdx5 exhibited a very weak EPR signal, preventing a definitive assignment. The observed resonances at approximately $g = 2$ and $g = 1.93$ align to some degree with those identified in Fdx11, which has been classified as adrenodoxin-type (*vide infra*). Reduced Fdx6 displayed a rhombic $S = \frac{1}{2}$ signal reflective of a [2Fe-2S] cluster; however, it is difficult to distinguish whether the signal reflects either a plant or adrenodoxin-type Fdx. The signal lacked axial-type symmetry that is a signature of other adrenodoxin-type Fdxs [49,50], however, it displayed a slightly lower degree of rhombicity (75%) compared to plant-type Fdxs 1–4 (Fig. 3, Table S3). Reduced Fdx10 and Fdx11 display $S = \frac{1}{2}$ signals with g -value anisotropy typical for thioredoxin-like [51] and adrenodoxin-type [2Fe-2S] cluster Fdxs, respectively [49,50].

Reduced Fdx7 and Fdx9 (EPR spectrum previously reported in Ref. [9] and shown here for comparison) display $S = \frac{1}{2}$ signals typical for [4Fe-4S] clusters [52,53] (Fig. 3). However, the spectrum of Fdx7 is more complex than is expected for a single, reduced, [4Fe-4S] cluster [5,33] and spectral fits identified two overlapping, reduced, [4Fe-4S] cluster signals with different rhombicity, g -values and relative contributions (Table S3). The structural basis and functional relevance of the heterogeneity of cluster signals is not immediately clear but may result from conformational variability in reduced Fdx7 leading to modulation of the Fe-S cluster electronic structure. It is possible there is a change in Cys coordination or Cys-SH H-bonding or cluster type (e.g., isomerization of [4Fe-4S] to [4Fe-3S]) but requires more extensive experimental validation. Compared to Fdx7, reduced Fdx9 displays an even more complex spectrum that is characteristic of $2 \times [4\text{Fe-4S}]$ magnetically interacting clusters. In this case, spin–spin coupling between the clusters is thought to give rise to the multitude of features in the $g = 2$ region, as well as line-shape broadening, as described previously [9].

Reduction of anaerobically prepared and reconstituted Fdx8 displayed a complex spectrum that could be simulated as two overlapping rhombic components consistent with two reduced [4Fe-4S] clusters (Figs 3 and S9). As noted above, the Fe-S cluster binding motifs of Fdx8 are predicted to coordinate a [3Fe-4S] cluster and a [4Fe-4S] cluster [5]. Possible conversion

of [3Fe-4S] clusters into [4Fe-4S] clusters in the reconstituted protein may explain this difference. This type of cluster conversion has been reported in detail for other [3Fe-4S], [4Fe-4S] di-cluster Fdxs prepared under reducing conditions [54–59]. Electronic interaction between the clusters may also contribute to the complexity of the spectrum, and for other di-cluster Fdxs, spin–spin coupling between clusters has been shown to lead to additional complexity of the $S = \frac{1}{2}$ region as observed here for Fdx8 [60,61]. This phenomenon may necessitate more advanced simulation, and the possible spin–spin coupling could account for features in the $g = 1.9$ region, as well as broadening on the wings of the spectrum that were not able to be replicated exactly in the simulation. Additional EPR experiments that investigated the effects of oxygen on Fdx8 suggest that one of the clusters, most likely coordinated by the three-cysteine motif, can alternate between a [3Fe-4S] and [4Fe-4S] (Table S4, Fig. S9). Experiments on Fdx8 specifically enriched in either [3Fe-4S], [4Fe-4S] or $2 \times [4\text{Fe-4S}]$ clusters are needed to determine the functional relevance of each type. From our work, it is not clear whether the oxygen induced cluster change in Fdx8 is functionally relevant or if it is simply a result of oxidative damage and warrants further investigations.

Comparative *in vivo* expression of Fdx1–Fdx11

To gain further information on Fdx protein expression levels under different growth conditions we constructed individual strains that expressed His-tagged versions of each Fdx (Fdx1 to Fdx11). Equivalent culture samples were separated by electrophoresis and used for immunoblotting with an antibody to the 6xHis-tag and visualized in side-by-side comparison (Figs 4 and S10).

We tested photoautotrophic (Fig. 4, panel A), photomixotrophic (Fig. 4, panel B), and heterotrophic (Fig. 4, panel C) conditions as well as nitrate (Fig. 4, panel E) and iron limitation (Fig. 4, panel F). In addition, growth under arginine and glucose (BG11₀ + Arg + Glc) culture conditions was tested (Fig. 4, panel D) as this promotes a highly reducing metabolism that requires HoxEFUYH expression for growth [62]. All Fdxs are detectable in at least one of the probed growth conditions. In all conditions, Fdx1 is by far the most dominant isoform as reported previously [5] followed by, typically, Fdx3 and Fdx5. Whereas Fdx2, Fdx3, Fdx4, Fdx5, Fdx9, Fdx10 and Fdx11 are expressed under all conditions but at different levels, Fdx6, Fdx7 and Fdx8 are expressed only at low levels or under detection limits under these conditions.

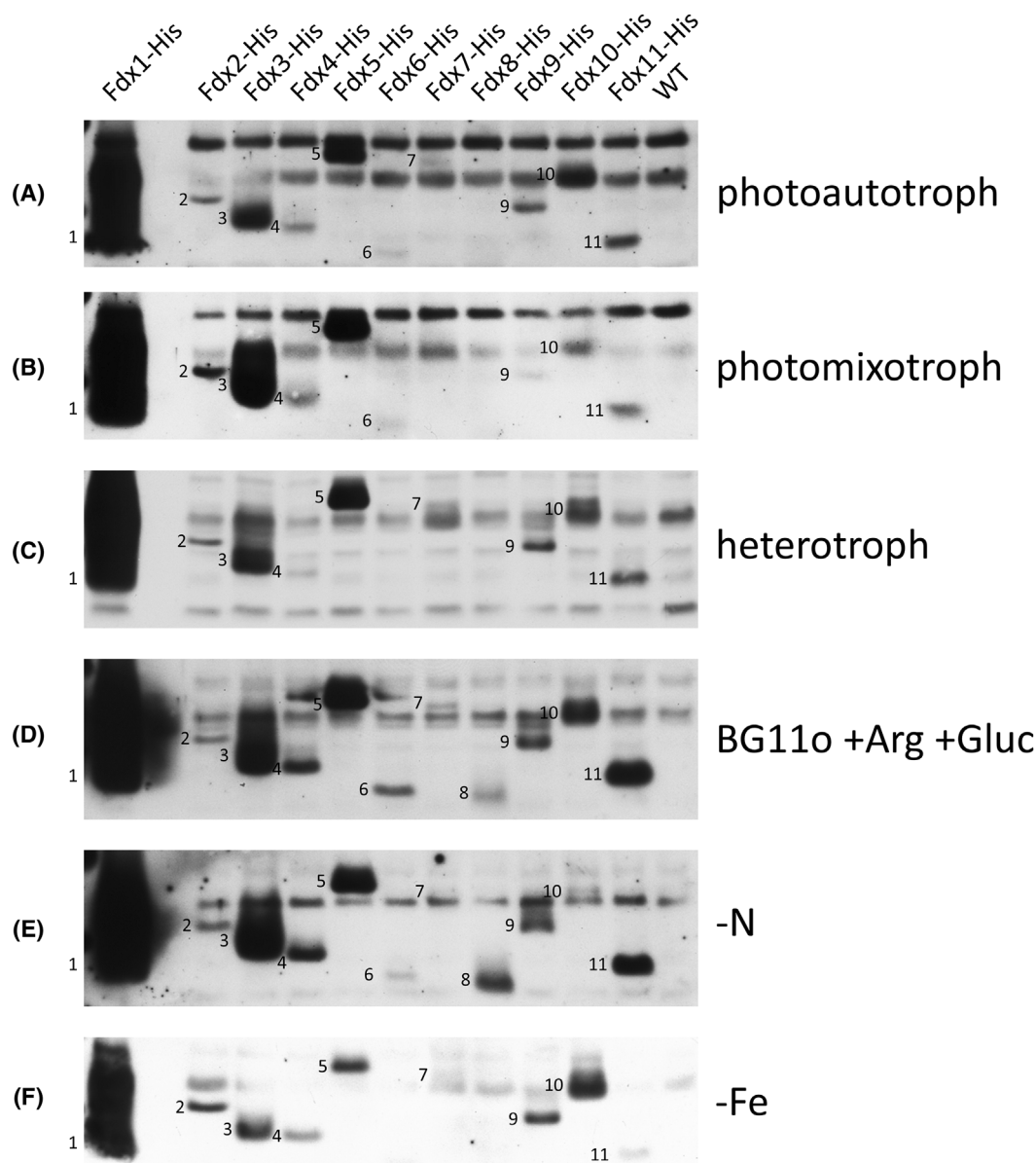


Fig. 4. Expression levels of His-tagged Fdxs 1–11 under various growth conditions assessed by immunoblotting with an α -His antibody (three different exposure times were typically developed; the numbers in the various panels correspond to the number of the respective ferredoxin and indicate the relevant antibody signal). Growth of cell material ($n = 1$) and sample preparation are outlined in the Materials and Methods section. Fdx reactive bands are marked with their respective number. It is important to note that different panels should not be compared quantitatively, based on apparent signal strength, since the amount of protein per lane is only equal within one condition (A) (photoautotroph (10 μ g), (B) photomixotroph (10 μ g), (C) heterotroph (7.5 μ g), (D) photomixotroph with arginine as nitrogen source (10 μ g), (E) nitrate depletion (1 μ g) and (F) Fe depletion (10 μ g)). General experimental limitations also have to be considered when comparing immunoblotting signal strengths.

Relative Fdx8 and Fdx4 expression levels were highest in cells grown under nitrate depletion (-N) or grown photomixotrophically with arginine as nitrogen source (BG11₀ + Arg + Gluc). Fdx4 and Fdx5 show distinctive expression levels even though they are organized in an operon and co-transcribed [28]. The expression

level of Fdx5 ranked among the highest under all growth conditions.

Fdx10 and Fdx11 are expressed under photoautotrophic conditions and may be involved in stress adaptation, since Fdx10 and Fdx11 have been shown to be upregulated under low temperature and Fdx11 is

upregulated under high-light and Fe-limitation [28]. Here we also observed that Fdx10 expression appears relatively stronger in cultures grown under heterotrophic, Fe-limitation, or in BG11₀ + Arg + Glc. Fdx11 expression also appeared relatively stronger in cells grown on BG11₀ + Arg + Glc or under N-limitation.

Determination of redox potentials

The formal midpoint redox potentials (E_m) of a Fdx contribute to the donor-acceptor free-energy for electron transfer with a protein partner. The E_m values of Fdx1–Fdx11 were investigated using square wave voltammetry (SWV), with the purified proteins immobilized on a pyrolytic graphite edge (PGE) working electrode (Table 1, Fig. S11). For all Fdxs, except Fdx9, a single redox peak was observed (Fig. S8) [9].

The E_m values of Fdxs1–11 were found to span a wide range of potential from –243 mV to –520 mV vs SHE (Table 1). The SWV analysis of Fdx5 produced an individual reduction potential peak that shifted in potential with repeated SWV scans, which suggests it may have reorientated on the PGE surface to result in different E_m values. It is also possible that this behavior arises from multiple cluster types or conformational heterogeneity. The E_m value of Fdx4 differed by approximately 100 mV when measured on a PGE versus a gold electrode. As the more negative E_m value is closer to values reported for a Fdx4 homolog measured in solution [45], this value will be considered further. It is important to note that E_m values obtained via protein film voltammetry can vary depending on the electrode material and may differ from those measured in solution. Such shifts in E_m values are likely protein-specific and may reflect differences in surface binding and conformational states on the electrode surface [3,63].

In vivo investigation on the interaction of nitrite reductase NirA with Fdxs1–12

Nitrite reductase (NirA, slr0898) is a redox enzyme that functions in nitrogen metabolism and based on relative E_m values, may couple reactivity to electron transfer with one of the Fdxs (Fig. S12 [8,14,64–69]). We tested this by examining the ability of a Fdx to form a binding interaction with NirA using a Nano-Luc luminescence complementation assay (Nanobit). The large subunit (nLucL) was fused to NirA and the small subunit, nLucS was fused to a Fdx, where a binding interaction results in a luminescence signal [70]. When Fdx1–11, nLucS fusions were expressed from their native promoters in strains grown under

photoautotrophic conditions, only Fdx1–nLucS produced a luminescence signal indicating an *in vivo* interaction with NirA–nLucL (Fig. S13). Since Fdx expression levels are highly regulated by growth conditions (Fig. 4), we constructed strains to bypass native regulatory control using rhamnose-inducible expression of Fdx–nLucS as well as IsiB–nLucS, (see Methods). Under the same growth conditions, the results also showed interactions between NirA–nLucL and Fdx4–nLucS and Fdx5–nLucS (Fig. 5). These binding interaction results with NirA and demonstration of Fdx4 and Fdx5 expression being elevated under nitrogen-limiting conditions or nitrogen source (Fig. 4) indicate a function in nitrogen metabolism by mediating electron transfer with NirA.

In addition to the NirA binding assay, we measured the biochemical reactivity of specific Fdx proteins with two of the complexes that have significant roles in *Synechocystis* photosynthetic metabolism; PSI ($F_B^- \rightarrow Fdx$, –580 mV, [68] and PFOR (–540 mV, [69] (Table 2)). Whereas PSI functions under photosynthetic growth conditions, PFOR functions under both highly reducing photomixotrophic and dark anaerobic metabolisms [27]. Both have relatively low redox potentials, and it is feasible, based solely on the free-energy requirements, that these complexes can participate in electron transfer and reduction of each Fdxs 1–11 (Table 2, Fig. S12).

In addition to the previously reported interaction of PSI with Fdx1 and Fdx4 [71], we demonstrate that Fdx1, Fdx3, Fdx4, Fdx8, and IsiB can function in electron transfer with PSI (Table 2, Fig. S14). Electron transfer from PSI was observed above the 30% electrons transferred threshold, but only for the 5:1 Fdx/PSI ratio sample in reactions with Fdx2, Fdx6 and Fdx7, suggesting that these Fdxs may not directly function in photosynthetic electron transport (Table 2, Fig. S14). Biochemical reactions with PFOR demonstrated that PFOR was able to reduce Fdx1, Fdx2, Fdx3, Fdx11 and IsiB, but not Fdx4, Fdx7 and Fdx10 (Table 2, Fig. S15). Fdx5, Fdx6 and Fdx8 were not tested in these reactions. Though not directly tested here, a summary of the Fdx reactivity profiles with HoxEFU and HoxEFUYH [8,16] is shown based on being required for growth on BG11₀ + Arg + Glc media. In agreement with the upregulation of Fdx 4 and Fdx11 under these conditions, they both support NAD⁺ reduction by HoxEFU, and Fdx4 (together with Fdx1, 2, 3, and IsiB) supports H₂ production by HoxEFUYH (Table 2). Overall, the activity results, together with the expression patterns (Fig. 4), identify that different Fdxs are important under different growth regimes. Fdxs 1–4 and 11 all showed electron

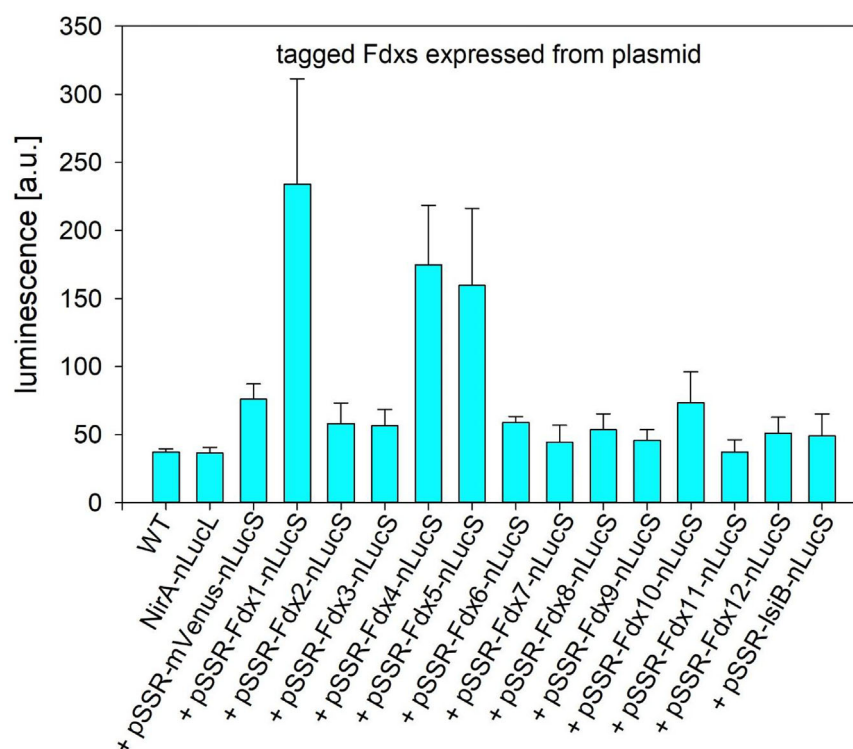


Fig. 5. Detection of *in vivo* interaction between NirA and Fdxs 1–12 by luciferase complementation in cells grown under photoautotrophic conditions. Strains with a genome encoded NirA-nLucL fusion (nLucL, luciferase large subunit) and transformed with a plasmid (pSSR) encoded rhamnose-inducible single Fdx-nLucS (Fdx, Fdx1–12; nLucS, luciferase small subunit). The bar is the luminescence level measured. Rhamnose-inducible mVenus fluorescent protein was expressed from pSSR as a negative control. Plots are the means and standard deviations of three independent experiments.

transfer activity that varied for the enzymes reported in Table 2.

Discussion

The diversity of the ferredoxin isoforms in *Synechocystis* serves as a specialized “electron circuitry” designed to maintain cellular redox homeostasis. While plant-type [2Fe-2S] ferredoxins are well-documented in cyanobacteria as the primary drivers of photosynthetic electron transport, this study illuminates the roles of the less understood adrenodoxin-type, thioredoxin-like, and bacterial-type isoforms. These proteins expand the function from distribution of photoelectrons and catalysis to sensing and metabolic regulation.

Fdx1 is an essential protein, strongly expressed across a range of growth conditions and supports electron transfer with a broad range of redox enzymes. Fdx3 is the second most abundant ferredoxin under the conditions tested and expressed at similar relative levels across growth conditions, except for during Fe-limitation (Fig. 4, Fig. S10). In contrast, Fdx4 and

Fdx8 are relatively higher expressed under nitrate depletion, while Fdx10 appears relatively more strongly expressed under iron limitation. Fdx4 (Slr0150) and Fdx5 (Slr0148) are encoded within the same operon and therefore polar effects from the insertion of the antibiotic resistance cassette into the genome of the respective His-tagged mutants cannot be excluded. Data from Kopf *et al.* [28] assigns both genes to one transcriptional unit with two transcriptional start sites upstream of slr0148, but none following downstream that would initiate an independent transcription of slr0150. Even though Fdx4 and Fdx5 are co-transcribed and Fdx4 has been shown to act as an electron transfer protein, Fdx5 did not have Fdx activity in reactions tested and shows divergent expression levels in this work. This suggests that *Synechocystis* utilizes post-transcriptional control to fine-tune the availability of specific isoforms in response to environmental signals.

Redox potentials range from -243 mV to -516 mV, suggesting that, thermodynamically, all isoforms, except for Fdx2, should support electron

Table 2. Summary of electron transfer reactivity of Fdxs and IsiB with PSI, PFOR, and HOX.

Protein	Reduction ^a and oxidation ^b reactions			
	PSI ^c $E_m = -580$ mV	PFOR $E_m = -540$ mV	HoxEFUYH $E_m = -413$ mV pH 7, 1 atm H ₂	HoxEFU $E_m = -320$ mV, NAD(P) ⁺ /NAD(P) (H)
Fdx1	+	+	+	+
Fdx2	+/-	+	+	+
Fdx3	+	+	+	n.d.
Fdx4	+	-	+	+
Fdx5	-	n.d.	n.d.	-
Fdx6	+/-	n.d.	n.d.	n.d.
Fdx7	+/-	-	n.d.	n.d.
Fdx8	+	n.d.	n.d.	n.d.
Fdx9	n.d.	n.d.	n.d.	n.d.
Fdx10	-	-	n.d.	n.d.
Fdx11	-	+	n.d.	+
Fdx12	n.d.	n.d.	n.d.	n.d.
IsiB	+	+	+	+

^aPSI reduction reactions kinetics (Fig. S14); Fdx1, Fdx4 and IsiB also from [71] and PFOR-Fdx reduction assays (Fig. S15); Fdx1-3, 11 and IsiB also from [27]; ^bPrevious studies of HoxEFU reactions were measured as either NAD(P)⁺ reduction coupled to Fdx oxidation, or NADH oxidation coupled to Fdx reduction [8,16]. HoxEFUYH production of H₂ from reduced Fdx1-4 and IsiB were measured in cell-free lysates [15]; ^c+, electron transfer was observed; -, electron transfer was not observed. +/-, reactivity was above a threshold of 30% of electrons transferred for the 5:1 Fdx:PSI ratio only (Fig. S14). n.d., not determined. Reactivities of Fdx9 and Fdx12 were not tested.

transfer to a broad range of dependent enzymes, including nitrite reductase (NirA). (Fig. S12), while PSI and PFOR could potentially function as electron donors. Furthermore, interactions with protein partners are determined by surface charge and kinetic gating, in addition to thermodynamic driving force. Plant-type Fdxs generally possess acidic (negative) surfaces that “steer” them toward positively charged docking sites on partners like PSI or FNR. Our *in silico* analysis revealed that Fdx10 and Fdx12 stand out with basic (positive) surface charges, an anomaly that suggests they evolved to interact with a unique set of acidic protein partners involved in specialized signaling rather than mainstream photosynthesis [28]. Unsuccessful reduction of Fdx5 is inconsistent with the relatively high Fdx5 redox potential value measured by square wave voltammetry (-280 mV). Our data suggest that the oxidation state of Fdx5 is likely dependent on conformational rearrangements that may regulate Fe-S cluster accessibility and impede its reduction [27].

Under photomixotrophic conditions, the simultaneous input of electrons from water splitting and glucose oxidation challenges cellular redox homeostasis. High NADH levels typically inhibit the pyruvate dehydrogenase (PDH) complex, leading PFOR to functionally replace PDH in carbohydrate catabolism. We experimentally verified that PFOR directly reduces Fdx3 *in vitro* and determined that Fdx3

($E_m = -464$ mV) and Fdx9 ($E_m = -520$ mV) possess the most negative redox potentials in the network. This low-potential capacity provides the thermodynamic prerequisite for their role as an electron sink, illustrating how *Synechocystis* leverages Fdx diversity to fine-tune redox homeostasis under extreme reducing pressure.

Fdx7 was previously reported to be involved in selenate tolerance [31]. In this context, it was shown to interact with FTR and glutaredoxin 2 (Grx2), the latter of which reduces selenate to less toxic forms [31]. The exact electron pathway of this Fdx-glutaredoxin-thioredoxin crosstalk to selenate involving Fdx7 was not clarified in detail. Our findings close this gap by suggesting that Fdx7 directly accepts electrons from PSI to be utilized for mitigation of oxidative and metal stress through the Fdx-glutaredoxin-thioredoxin pathway.

Until now, only scarce information has been available regarding the function of Fdx8 other than it is essential for survival [5,27] and that its expression is elevated under Fe-limitation and high-light [28]. Our results demonstrate that Fdx8 expression is relatively highest under nitrogen depletion conditions which affect the cell capacity to catalyze nitrate reduction and carbon fixation. Interaction with PSI (Table 2) suggests that Fdx8 might be involved in coupling photosynthetic electron transport to other terminal acceptors to maintain energy balance under nitrogen limitation.

While Fdx8 is essential for survival, its expression could not be detected under most of the tested conditions, only under nitrogen starvation and also in BG11₀ + Arg + Glc, suggesting that it serves a vital role during nutrient-induced stress. Although the Fe-S cluster binding motifs of Fdx8 predict the coordination of a [3Fe-4S] and a [4Fe-4S] cluster [5], we confirmed experimentally the presence of 2×[4Fe-4S] clusters, the level of which was dependent on oxygen conditions. Fdx8 may fine-tune its electron transfer properties as oxygen levels change. For example, one of the two forms could either be inactive or confer different reaction kinetics of Fdx8 with redox partners (e.g., PSI). Along this line, aconitase, an enzyme of the TCA cycle that catalyzes the conversion of citrate to isocitrate, contains a [4Fe-4S] cluster which can reversibly form a [3Fe-4S] cluster leading to a change in protein function as a mRNA binding iron regulatory protein [54]. The newly identified Fdx10 is a thioredoxin-type ferredoxin. Its classification and inability to accept electrons from PSI, combined with its unique basic surface charge, strongly indicates a role in redox-dependent protein regulation and stress-sensing rather than linear electron flux. Finally, Fdx11 expression is relatively strongest under nitrogen starvation and also in BG11₀ + Arg + Glc. Fdx11 supports electron transfer from PFOR and NADH reduction catalyzed by the [NiFe]-hydrogenase diaphorase HoxEFU complex, thereby facilitating metabolic adaptation under highly reducing conditions, such as photomixotrophic growth.

Ultimately, the *Synechocystis* Fdx suite represents a division of labor between high-volume photosynthetic electron flux and specialized metabolic regulation. By moving beyond the well-known plant-type proteins, we demonstrate how this suite of specialized ferredoxins allows the cell to balance the competing demands of efficient energy harvest and robust stress protection. Even though it was our aim to present a comprehensive study on the ferredoxins of *Synechocystis*, more work is needed, for example, a closer look at the available knock-out mutants or further *in vivo* interaction experiments, to unravel all the roles and functions of the ferredoxins.

Materials and methods

Sequence retrieval

Previous studies reported on nine Ferredoxins in *Synechocystis* [5]. Here we identified proteins Fdx10–12 in database searches on webpages (CYANOBASE, <http://genome.kazusa.or.jp/cyanobase>; CYORF, <http://cyano>.

genome.jp/; both have been discontinued in the meantime) (Table 1). Amino acid sequences were obtained from KEGG and UniProtKB/Swiss-Prot protein databases (Fig. S1).

Computational structure analysis

Fdx structures and Fe-S cluster binding sites were predicted using AlphaFold2 (AF2) [72] and AlphaFill [73]. The predicted structures were further refined using the AMBER force field. To assess structural and sequence similarities, proteins with significant homology to each ferredoxin were identified based on the Global Model Quality Estimate (GMQE) score from SWISS-MODEL, with scores above 0.5 considered significant [74]. Protein surface charges were calculated using PyMOL and the APBS surface charge calculation plugin.

Construction of expression and mutagenesis plasmids

Synechocystis ferredoxins were heterologously expressed in *E. coli*. The proteins were expressed from plasmids that added a TEV cleavage site containing linker [75] and either a tandem GST-6xHis-tag (Fdx1, Fdx2, Fdx3, Fdx4, Fdx5, Fdx6, Fdx7, Fdx9, Fdx10, Fdx11, Fdx12, IsiB) [76,77] or a tandem MBP-6xHis-tag (Fdx6, Fdx8, Fdx9, Fdx10) to the C-terminus. To generate the GST-6xHis expression plasmids, the genes of interest were amplified from *Synechocystis* WT genomic DNA using the primers 1 to 26 (Table S4) and cloned into a previously described plasmid [77] that had been opened with *NdeI* and *BamHI*. In some cases, the obtained PCR fragments were subcloned into the pGEM®-T easy vector (Promega), while in others the fragments were directly inserted into the expression vector via Gibson cloning [78]. To be able to generate the MBP-6xHis expression plasmids, some prior modifications to the GST-6xHis expression vector were required. By opening the vector with *NdeI* and *EcoRI*, only the pRSETA backbone was used. A fragment containing overlapping ends that allowed an initial insertion into the pBluescript SK+ plasmid (digested with *KpnI* and *SacI*) via Gibson cloning was ordered (GenScript, Fig. S16). The ordered fragment also contained the sequence encoding *Synechocystis* Fdx9, a 17 amino acid (aa) linker (adapted from [75]) with an internal TEV cleavage site followed by a *SmaI* site, a 6xHis-tag, a stop codon (TAA), an *EcoRV* site, another stop codon (TAG) and an *EcoRI* site. It was excised again from the pBluescript vector using *NdeI* and *EcoRI* and inserted into the previously opened GST-6xHis expression vector yielding plasmid pMB0122. The MBP encoding sequence was amplified using primers 27 and 28 (Table S4) from the pRK793 plasmid (Addgene) and subcloned into the pGEM®-T easy vector (Promega). After excision with *NheI* and *SmaI* from the resulting pGEM®-T easy vector, the MBP-tag encoding

sequence was inserted into the *NheI* and *SmaI* opened pMB0122 plasmid. For these expression vectors codon-optimized versions of previously difficult to express ferredoxins (Fdx6, Fdx8, Fdx9, Fdx10; Fig. S17) were ordered (Genscript) and could be inserted into the pMB0122 plasmid using the *NdeI* and *BamHI* restriction sites. All expression plasmids were sequenced (Azenta).

For the nanoLuc *in vivo* interaction studies between nitrite reductase and Fdxs, the LgBit luciferase (nLucL) coding fragment together with a gentamycin resistance cassette was excised from a toolkit vector (pMB00071) using *BamHI* and *EcoRI*, ligated into the *BamHI* and *EcoRI* opened NirA C-entry plasmid (ordered from Azenta; NirA contains an internal *BamHI* site that was silently mutated in this C-entry vector) and transformed into *Synechocystis*. The resulting mutant was confirmed by PCR using primers 75 and 76 (Table S4) and then transformed either with plasmids that (A) added the SmBit luciferase (nLucS) together with a spectinomycin cassette (*BamHI* and *EcoRI* excised from the toolkit vector pMB0070) before the STOP codon of the investigated Fdxs (using the same C-entry vectors mentioned above) or (B) with a rhamnose-inducible plasmid for the expression of a respective ferredoxin tagged with the SmBit luciferase (nLucS) tag. These plasmids derived from a plasmid that has been described in the literature [79] and into which the SmBit coding region was introduced, so that a gene of interest could be incorporated into the vector using a *NdeI* restriction site. The primers used to generate the Fdx fragments that were incorporated into this vector via Gibson cloning were primers 77 to 100 (Table S4). Mutants were confirmed by PCR using the same primers as for the His-tagged strains for (A) and primers 103 and 104 for (B) (Table S4).

Protein expression and preparation

Table S2 lists the expression plasmids and strains that were utilized for each of the purified proteins. For each Fdx6, Fdx9, and Fdx10, several cell cultures were grown independently, and later matching cell material was combined for protein purification. The KRX strain (Promega) does not harbor an antibiotic resistance, and protein expression was induced by the addition of 0.05% (w/v) L-rhamnose. The RosettaTM 2 pLysS (DE3) strain (Novagen) harboring the pRARE2 plasmid is resistant to chloramphenicol (25 µg·mL⁻¹) and expression was induced by the addition of 0.5 mM IPTG. The C41 strain harboring the pCodonPlus and pRKISC plasmids [80,81] is resistant to chloramphenicol (25 µg·mL⁻¹) and tetracycline (10 µg·mL⁻¹), and expression was induced by the addition of 0.5 mM IPTG. The heterologous expression of Fdx8 was carried out in ΔiscR BL21 [43] to stimulate maturation of FeS-clusters in recombinant protein in a form that is biologically relevant. Fdx8 was expressed and purified under either aerobic or strictly anaerobic conditions. Fdx9 was expressed as described previously [9].

The expression plasmids used in this study conferred ampicillin resistance (100 µg·mL⁻¹). A previously established method [76,77] was modified and employed for protein preparation. In brief, a 5-mL LB starter culture was grown overnight at 37 °C and diluted 1 : 100 in one or several 100-mL LB subculture the following morning. After approximately 3 h of growth at 37 °C, 20 mL of the culture were used to inoculate 2 L-flasks containing 1 L of LB. All cultures contained appropriate antibiotics and were grown aerobically. At an OD₆₀₀ of around 0.7, protein production was induced by adding the appropriate inducing agent and 1 mM ferric ammonium citrate prior to a shift to 20 °C. Cells were harvested the following morning (15 min, 4 °C, 5000 × g) and resuspended in lysis buffer (50 mM NaPO₄ pH = 7.0, 250 mM NaCl) for breakage by sonication (Sontrode MS73 (Bandelin); 8 repeats of 20 s on (70% cycle, 70% power) and 20 s off). The supernatant obtained after ultracentrifugation (45 min, 4 °C, 100 000 × g) was incubated for 1 h at 4 °C with TALON[®] SuperflowTM resin (Takara). After the incubation period, the resin was washed with 20 column volumes (CV) of lysis buffer or until the wash solution became clear and colorless. Protein elution was performed with 5 CV of elution buffer (50 mM NaPO₄ pH = 7.0, 250 mM NaCl, 250 mM imidazole). The eluted proteins were dialyzed overnight in 50 mM Tris pH = 8.0, 100 mM NaCl in the presence of TEV-His protease His-tag purified from pRK193 (Addgene) [82]. The following day, the protein was incubated again with TALON[®] SuperflowTM resin (Takara) and the flow-through was collected, concentrated and loaded onto a HiLoadTM 26/60 SuperdexTM 75 prep grade (Cytiva) using a previously established method [77]. The run was monitored at 280 nm, 2-mL fractions were collected and the final protein concentrations were determined using ROTI[®]Nanoquant assay (Roth) according to the manufacturer's instructions.

Protein analyses

To assess the purity of heterologously expressed protein after purification, some sample was mixed with 2× Laemmli sample buffer, boiled for 5 min at 95 °C and centrifuged in a microfuge (5 min, max speed). Roughly 5 µg of the purified protein were then loaded per lane and separated on a 15% (w/v) polyacrylamide BisTris gel using MES running buffer (250 mM MES, 250 mM Tris, 5 mM EDTA, 0.5% (w/v) SDS). To probe ferredoxin expression levels, aliquots corresponding to 50 mL of the respective cell cultures were resuspended in 500 µL of ACA buffer (750 mM ε-aminocaproic acid, 50 mM BisTris/HCl, pH 7.0, 0.5 mM EDTA). Cells were broken by vortexing with ~200 µL glass beads (0.17–0.18 µm diameter; Sigma) for 2 min at full speed at 4 °C. Glass beads and cell debris were then pelleted by centrifuging in a microfuge at full speed for 1 min. The whole cell extract (WCE) was centrifuged again at 20 000 × g for 20 min, the supernatant was

kept as the soluble extract and its protein concentration was determined using the ROTI-Quant protein Bradford assay according to the manufacturer's instructions (Carl Roth). Due to the fact that the amount of cell material and cell breakage were different for different samples and conditions equal loading within one dataset (growth condition) was ensured by varying amounts of loaded protein (autotroph (10 µg), mixotroph (10 µg), heterotroph (7.5 µg), mixotroph with arginine as nitrogen source (10 µg), nitrate depletion (1 µg) and Fe depletion (10 µg)) that were loaded per lane on a 17.5% (w/v) polyacrylamide BisTris gel using MES running buffer (250 mM MES, 250 mM Tris, 5 mM EDTA, 0.5% (w/v) SDS). Samples were denatured with 2× Laemmli sample buffer for 1 h at RT. Gels were Coomassie-stained or electroblotted onto nitrocellulose membrane. For immunoblotting, 5% (w/v) milk powder in 1× PBS-T was used as blocking solution and all washing steps were performed with 1× PBS-T. Immunoblot analyses were performed using THE™ His-tag antibody (Genscript) and horseradish peroxidase-conjugated secondary antibodies (anti-mouse, Cytiva).

LC/MS analysis

For protein identification following purification, 0.5 µL of each sample, containing 2–14 µg of protein, was mixed with SDS loading buffer and heated at 95 °C for 5 min. As a preliminary cleaning step, the boiled samples were run into a 4% SDS/PAGE stacking gel at 120 V. After the entire sample exited the well and was captured within the gel, the proteins were excised using an ethanol-cleaned razor blade. The excised gel pieces were prepared for protein mass spectrometry via a nano-liquid chromatography tandem mass spectrometer (nLC-MS/MS) through a series of steps: destaining, reduction of disulfide bridges, cysteine carbamidomethylation, and tryptic in-gel digestion, following an established protocol [83]. The resulting peptides were extracted in multiple steps using 50% (v/v) acetonitrile, 0.1% (v/v) formic acid, or 5 mM ammonium bicarbonate, and subsequently desalted using ZipTip-µC₁₈ material (Merck Millipore). After desalting, the peptides were dried in a vacuum centrifuge and resuspended in 20 µL of 0.1% (v/v) formic acid before mass spectrometry analysis on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) coupled to an nLC system Dionex Ultimate 3000RSLC (Thermo Scientific) via an electrospray ion source TriVersa NanoMate (Advion). A 5-µL volume containing the peptides was separated on an Acclaim Pep-Map100 C₁₈ column (Thermo Scientific) at a flow rate of 300 nL·min⁻¹, following the parameters described elsewhere [84].

Proteome Discoverer v2.2 (Thermo Scientific) was employed for protein identification using the SequestHT search engine against the proteome databases of the production strains *E. coli* K12 (taxonomy ID: 83333), used for

the heterologous production of Fdx1 to Fdx11, or *E. coli* BL21 (DE3) (taxonomy ID: 511693), used for the production of IsiB, from the UniProt database, supplemented with the amino acid sequences of the target proteins (Fdx1–Fdx11 and IsiB). The following parameters were set: carbamidomethylation of cysteine residues as a fixed modification, oxidation of methionine as a dynamic modification; trypsin cleavage specificity with a maximum of two missed cleavage sites; precursor and fragment mass tolerances set to 3 ppm and 0.6 Da, respectively. Stringent identification criteria were applied with a 1% false discovery rate threshold for peptide identification using the Target Decoy PSM Validator node. Protein quantification was facilitated by the Minora node in Proteome Discoverer through label-free quantification based on MS1 precursor intensity values. Relative protein abundance was calculated by normalizing the MS1 intensities of the target proteins in the gel slices to the overall MS1 intensities of all detected proteins within the same slices.

UV/visible absorption spectra

The spectra of the different ferredoxins and flavodoxin were measured in the range from 300 to 600 nm using a Cary-60 UV-Vis photometer (Agilent, CA, USA). In a total volume of 1 mL of 100 mM Tris pH = 8, a protein concentration that gave a reading in its maximum of at least 0.3 was used. After subtraction of the baseline, this was used as the oxidized spectrum. After addition of 40 µL of freshly prepared 100 mM sodium dithionite to the same sample, the reduced spectrum was recorded. Since dithionite gives a strong reading below 380 nm, only the spectra above this wavelength are shown.

Electron paramagnetic resonance spectroscopy

EPR samples of reduced ferredoxins 1–7 and 10–11 (100 µM final concentration) were prepared in 25 mM NaPO₄ pH 7.0, 50 mM NaCl, 5% glycerol under anaerobic conditions in an MBRAUN glovebox (nitrogen atmosphere). Samples were reduced with NaDT (5 mM final concentration) and loaded into EPR tubes (Wilmad 707-SQ-250 m), which were sealed with septa and frozen in liquid nitrogen. Fdx8 was reconstituted according to the procedure previously described for Fdx9 [9]. A reduced sample for Fdx8 was prepared by reduction with 10 mM NaDT in 50 mM Tris pH 8.3, 200 mM NaCl, 5% glycerol. EPR was collected on a Bruker Elexsys E500 spectrometer as previously described [9]. Spectra were processed in OriginPro 2024b by baseline correction through subtraction of a spline function and background subtraction of buffer. Simulations of the EPR spectra were carried out in the EasySpin toolbox [85] using the “esfit” least-squares fitting algorithm and EasySpin “pepper” function in MATLAB R2023b. In Fig. 3, the EPR spectra signal intensity was

normalized to the peak height of the signal corresponding to first *g*-value for Fdxs 1–4, 6–8, 10–11 and to the peak height of the middle of the spectrum for Fdx9. The EPR signal intensity of Fdx5 was magnified by a factor of 5.

To investigate the nature of the Fdx8 Fe-S clusters and the impacts of oxygen, we conducted EPR experiments on Fdx8 expressed under aerobic and strictly anaerobic conditions in the “as purified” state or reduced with 5 mM sodium dithionite (Fig. S9). These samples were not reconstituted, in contrast to the EPR spectrum of reduced Fdx8 displayed in the main text (Fig. 3), as well as previous studies [55,86]. Generally, EPR signals of oxidized [3Fe-4S] clusters can be detected while reduced [3Fe-4S] clusters are EPR silent. The opposite is true for [4Fe-4S] clusters, where EPR signals of reduced [4Fe-4S] clusters can be detected and oxidized [4Fe-4S] clusters are EPR silent.

Expression level analysis

To probe ferredoxin expression levels under different growth conditions, 6xHis-tagged mutant strains for ferredoxins 1 to 11 were generated. Each mutagenesis plasmid was generated from a C-entry vector (opened with *Bam*HI and *Eco*RI) into which a cassette excised from a toolkit vector (with *Bam*HI and *Eco*RI) was ligated. The C-entry plasmids used the pBluescript SK+ plasmid (digested with *Kpn*I and *Sac*I) as a backbone and contained two overlapping ~250 bp DNA fragments (up- and downstream of the respective stop codon of the gene of interest) that were introduced via Gibson cloning. The primers 29 to 72 (Table S4) used to generate the two fragments further introduced the following bases 5'-CCCATCGGA-3' followed by *Bam*HI, *Xho*I and *Eco*RI restriction sites directly before the stop codon. The toolkit vector for the 6xHis-GENT cassette was constructed by inserting a PCR amplified gentamycin resistance cassette (primers 73 and 74) by blunt-ended ligation into the *Eco*RV site of pMB0122. The final mutagenesis plasmids were sequenced (Azenta) and transformed into the *Synechocystis* wild-type strain to create the 6xHis-tagged ferredoxin mutant strains. Mutant segregation was confirmed by PCR (e.g., using primer 29 and 32 for Fdx1). *Synechocystis* cultures to probe ferredoxin expression levels were grown under the following conditions: autotroph (BG11; start $OD_{750} = 0.03$; 5 days; mean end $OD_{750} = 0.798 \pm 0.122$), mixotroph (BG11, 10 mM glucose; start $OD_{750} = 0.2$; 2 days; mean end $OD_{750} = 1.767 \pm 0.066$), heterotroph (dark; BG11, 10 mM glucose; start $OD_{750} = 0.2$; 2 days; mean end $OD_{750} = 0.483 \pm 0.065$), mixotroph with arginine as nitrogen source (BG11₀, 10 mM glucose, 5 mM arginine; start $OD_{750} = 0.15$; 3 days; mean end $OD_{750} = 1.176 \pm 0.216$), nitrate depletion (BG11₀; start $OD_{750} = 0.15$; 3 days; mean end $OD_{750} = 0.384 \pm 0.048$) and Fe depletion (BG11 -Fe; start $OD_{750} = 0.2$; 3 days;

mean end $OD_{750} = 0.776 \pm 0.115$) [87]. Starting OD_{750} and duration of growth differ between growth conditions, but were adapted to obtain sufficient cell material for subsequent analyses. No claims are based on the growth phase of the harvested cultures and OD_{750} values are given for transparency. In each case, 50-mL pre-cultures with appropriate antibiotics were started four days ahead of the experiment and cultivation started after washing the pre-cultures with the target growth medium (without antibiotics) and adjusting the start OD_{750} to the indicated values. Cell growth occurred in one Kniese tubes per strain (WT, Fdx1-6xHis to Fdx11-6xHis) in a volume of 150–200 mL liquid culture with constant ambient air bubbling at 28 °C and 50 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. Cells were harvested after the indicated durations and subjected to protein analyses.

Square wave voltammetry

Protein film square wave voltammetry (SWV) was performed in an MBraun anaerobic chamber according to previously published protocols [8]. Square wave voltammetry (SWV) measurements were performed in a standard three-electrode cell using a pyrolytic graphite edge (PGE) working electrode, an Ag/AgCl (saturated KCl) reference electrode, and a platinum wire counter electrode. The working electrode was polished before each experiment, and background signals were recorded in buffer without protein. Protein films were incubated on the electrode for at least 10 min prior to measurements in a buffer containing 150 mM HEPES, 200 mM NaCl, and 5% glycerol at pH 8. The same buffer was used as the electrolyte. SWV was conducted using a CH Instruments 630C potentiostat with a 0.001 V step size, 0.025 V amplitude, 10 Hz frequency, and 1 $\mu\text{A/V}$ sensitivity. Current and potential were simultaneously sampled at 1 MHz. All potentials were converted to values versus the standard hydrogen electrode (SHE) by adding 199 mV to the Ag/AgCl reference.

NanoLuc interaction studies

Strains to assess the interaction between nitrite reductase and Fdxs were grown under autotrophic growth conditions. Cells were pelleted by centrifugation and the OD_{750} was normalized to 1. 200 μL of normalized cell culture were transferred into three wells of a white 96-well plate with a transparent bottom and 50 μL of 25 μM Coelenterazine as substrate were added. After transferring the plate into an Infinite M Plex plate reader (Tecan) and reaching 30 °C in the inner chamber, OD_{750} was measured followed by a recording of the luminescence for 30 min (integration time 2 s). The luminescence signals were averaged per well and normalized according to measured OD_{750} . Then the signal was averaged for each strain and plotted.

P700⁺ reduction kinetics

PSI protein complexes employed for the P700⁺ reduction kinetics measurements were purified from a PsaF-6xHis mutant strain constructed as described elsewhere [88] using a previously described protocol [89,90] with modifications. Briefly, a 30-L *Synechocystis* PsaF-6xHis culture was harvested (15 min, 4 °C, 5000 × *g*) and resuspended in buffer A (20 mM MES pH 6.5, 10 mM MgCl₂, 10 mM CaCl₂). Cells were broken using a multi-cycle cell disruptor at 35 kpsi (Constant Cell disruption Systems). Membranes were pelleted (10 min, 4 °C, 20 000 × *g*), washed in buffer A, pelleted (10 min, 4 °C, 20 000 × *g*), washed in buffer B (20 mM MES pH 6.5, 10 mM MgCl₂, 10 mM CaCl₂, 500 mM mannitol) to which fresh 0.05% (w/v) beta-dodecyl-D-maltoside was added and pelleted again (10 min, 4 °C, 20 000 × *g*). Subsequently, the membranes were resuspended in extraction buffer (20 mM HEPES pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 200 mM ammonium sulfate), the Chl *a* concentration adjusted to 1 mg·mL⁻¹ and the membranes solubilized with 0.5% (w/v) N,N-Dimethyl-n-dodecylamine N-oxide (LDAO) for 30 min at RT with gentle agitation. After pelleting insoluble material (60 min, 4 °C, 100 000 × *g*), the supernatant was applied to a prepacked fast flow Co-TALON resin cartridge (Takara) connected to an Akta pure (Cytiva) with (20 mM HEPES pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 5 mM imidazole, 0.03% (w/v) beta-dodecyl-D-maltoside) as the running buffer. The sample was washed with 10 CV running buffer and after elution with (20 mM HEPES pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 100 mM imidazole, 0.03% (w/v) beta-dodecyl-D-maltoside) green fractions were pooled, concentrated and the Chl *a* concentration determined. Prior to flash freezing and storage at -80 °C the PSI protein complexes were mixed 1:1 with a PSI storage buffer (20 mM HEPES pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 1 M mannitol, 0.03% (w/v) beta-dodecyl-D-maltoside).

Electron transfer from PSI to various Fdxs was assessed by monitoring P700⁺ re-reduction kinetics using a Dual-KLAS/NIR instrument [91]. Assays (1 mL) contained 500 nM PSI (based on Chl *a* content), 500–2500 nM Fdx, and an oxygen-scavenging system 10 mM glucose, 20 U glucose oxidase and 25 U catalase in activity buffer (25 mM Tris-HCl buffer pH 8.0, 10 mM CaCl₂, 10 mM MgCl₂ and 0.03% (w/v) beta-dodecyl-D-maltoside). Following a 5-min dark incubation, the sample was subjected to a 10 ms multiple-turnover light pulse (13 600 μE·m⁻²·s⁻¹). In the absence of an acceptor, P700⁺ re-reduction is rapid due to charge recombination, that is, successful electron transfer to an Fdx slows this re-reduction rate. PSI alone (no transfer) and PSI with 1 mM methyl viologen (complete transfer) served as benchmarks. The P700⁺ re-reduction slope was calculated over the first 100 ms post-illumination. Slopes were normalized against signal amplitude (pre- vs. post-pulse) and compared to the PSI-only control to determine the percentage of electron transfer.

PFOR activity assay

To assay potential interactions between the various ferredoxins and the pyruvate ferredoxin oxidoreductase (PFOR), the method reported previously [27] was adapted. Briefly, an assay with a final volume of 1 mL containing 4.5 mM ferredoxin, 270 μM PFOR, 0.5 mM coenzyme A, 10 mM pyruvate, 5 mM thiamine pyruvate phosphate as well as 40 mM glucose, 40 U glucose oxidase and 50 U catalase (to maintain anaerobiosis) in 100 mM Tris-HCl buffer pH 8.0 was incubated for 30 min at room temperature (25 °C) while absorption at 460 nm was monitored using a Cary 60 UV-Vis photometer (Agilent). The assay was started by the addition of the respective ferredoxin at concentrations up to 100 μM.

Author contributions

MB, DS, LA, PWK and KG designed research; MB, DS, CEL, JA, DWM, EK, VH, KS, VSB, ZG, AEL and DD performed research; all authors analyzed data; and MB, DS, CEL, DWM, ECK, PWK and KG wrote the paper.

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During the preparation of this work the author(s) used ChatGPT (Open AI 2025), ChatGPT Team (5.1) and DeepL in order to optimize language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article. Open Access funding enabled and organized by Projekt DEAL.

Conflict of interest

The authors declare no conflict of interest.

Peer review

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Data availability statement

The supporting data for this manuscript are provided as [Supporting Information](#).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Amino acid sequences of Fdx1–Fdx12 from *Synechocystis* sp. PCC 6803.

Fig. S2. Structures of Fdx5, Fdx6 and Fdx11.

Fig. S3. AlphaFold-predicted structure of Fdx7, highlighting a poorly defined N-terminal region (yellow) with low confidence.

Fig. S4. Sequence alignment of Fdx8 with selected other [7Fe-8S] and [8Fe-8S] ferredoxins with available crystal structures.

Fig. S5. Fdx8 structural model showing Cysteine residue distribution, as well as the position of Val13 in proximity to the [3/4Fe-4S] cluster.

Fig. S6. SDS/PAGE analysis of the purified *Synechocystis* Fdx1-Fdx11 and flavodoxin (IsiB) (see also Table S2).

Fig. S7. UV-Vis absorption spectra of as purified Fdxs 1-11 and flavodoxin (black line), as well as spectra from reduced proteins (red dashed line) between 350 and 450 nm.

Fig. S8. (A) UV-Vis spectra of Fdx1, Fdx7, and Fdx8.

Fig. S9. EPR spectra of Fdx8 expressed and purified under aerobic or strictly anaerobic conditions.

Fig. S10. Qualitative analysis of Fdx expression levels for the immunoblot analysis shown in Figure 4.

Fig. S11. Redox potentials of ferredoxins.

Fig. S12. Comparison of redox midpoint potentials (E_m) for *Synechocystis* Fdxs and redox potentials (E_m) of reactions catalyzed by relevant protein partners.

Fig. S13. *In vivo* interaction of nitrite reductase with Fdxs1-11.

Fig. S14. Electron transfer from photosystem I (PSI) to purified Fdxs.

Fig. S15. Electron transfer from pyruvate ferredoxin oxidoreductase (PFOR) to purified Fdxs.

Fig. S16. Fragment ordered from Genscript for the generation of the pMB0122 vector.

Fig. S17. Codon-optimized sequences of various Fdxs (Genscript).

Table S1. A comparison of the surface charges on all *Synechocystis* Fdxs studied in this work.

Table S2. Information on Fdx1 to Fdx12 and flavodoxin (IsiB) over-expression and purification.

Table S3. EPR properties of the reduced *Synechocystis* Fdx signals presented in Fig. 3.

Table S4. Primers used in this work.