The structure and reactivity of the HoxEFU complex from the cyanobacterium *Synechocystis* sp. PCC 6803

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**Abstract:** Cyanobacterial HOX is a [NiFe] hydrogenase that consists of the hydrogen (H2)-activating subunits HoxYH, which form a complex with the HoxEFU assembly to mediate reactions with soluble electron carriers like NAD(P)H and ferredoxin (Fdx), thereby coupling photosynthetic electron transfer to energy-transforming catalytic reactions. Researchers studying the HoxEFUYH complex have observed that HoxEFU can be isolated independently of HoxYH, leading to the hypothesis that HoxEFU is a distinct functional subcomplex rather than an artifact of HOX complex isolation. Moreover, outstanding questions about the reactivity of HOX with natural substrates and the site(s) of substrate interactions and coupling of H2, NAD(P)H, and Fdx remain to be resolved. To address these questions, here we analyzed recombinantly produced HoxEFU by EPR spectroscopy and kinetic assays with natural substrates. The purified HoxEFU subcomplex catalyzed electron transfer reactions among NAD(P)H, flavodoxin, and several ferredoxins, thus functioning in vitro as a shuttle among different cyanobacterial pools of reducing equivalents. Both Fdx1-dependent reductions of NAD+ and NADPH were cooperative. HoxEFU also catalyzed the flavodoxin-dependent reduction of NAD(P)+, Fdx2-dependent oxidation of NADH, and Fdx4- and Fdx11-dependent reduction of NAD+. MS-based mapping identified an Fdx1-binding site at the junction of HoxE and HoxF, adjacent to iron–sulfur (FeS) clusters in both subunits. Overall, the reactivity of HoxEFU observed here suggests that it functions in managing peripheral electron flow from photosynthetic electron transfer, findings that reveal detailed insights into how ubiquitous cellular components may be used to allocate energy flow into specific bioenergetic products.

**Introduction**

*Synechocystis* sp. PCC 6803 (S. 6803) has long served as a model photosynthetic organism to address questions on the biochemistry and mechanisms of photosynthetic electron transfer, including the function of peripheral redox enzymes that maintain redox homeostasis (1,2). In *S.* 6803 one example is the bidirectional hydrogenase, HoxEFUYH that, depending on the metabolic context, either functions to dispense with excess reducing power through the generation of hydrogen, or couples the oxidation of hydrogen to the reduction of electron carrier pools (3,4). HoxEFUYH has long been of interest due to the possibility of engineering the enzyme and metabolic pathways to enable photobiological hydrogen production routes (5). Despite numerous investigations, the underlying features of the biochemistry, structure, and protein-protein interactions of HoxEFU have yet to be fully detailed. A more complete understanding of these
properties could be used to improve approaches for engineering photobiological hydrogen production, artificial photosynthesis, or catalyst design.

HoxEFUYH consists of the small and large [NiFe] hydrogenase subunits, HoxY and HoxH, respectively, and an additional set of subunits that comprise the heterotrimeric diaphorase, HoxEFU (6) (Fig. 1). Biochemical reactions with ferredoxins 1 and 4 (Fdx1 and Fdx4, ssl0020 and slr0150, respectively), flavodoxin (Fld), and NAD(P)H demonstrated that each of these was capable of supporting catalytic H₂ production by HOX in cell extracts (4,7,8) and purified enzyme (9) (Table S1). Studies with the intact HoxEFUYH heteropentamer have further suggested there is a preference for NADH over NADPH (Table S2) (4,7,9-12). The ability of HoxEFUYH to couple H₂ oxidation to NAD(P)⁺ reduction is less clear, however. In cell extracts, this activity was either undetectable (7) or barely detectable (8), and in whole cells where H₂ oxidation was observed, the electron acceptor was not definitively identified (13). The reactivity of purified HoxEFU for NAD(P)⁺ reduction has not yet been clearly shown and may differentiate S. 6803 HoxEFUYH from other related enzymes that have more readily reversible diaphorase activity (10,11,14), and in particular for HOXs (HoxYH with or without the diaphorase subunit) from non-photosynthetic bacteria which can have different reactivities and subunit compositions.

The HoxEFU sub-complex contains a complex array of redox cofactors. Whereas HoxYH harbors the [NiFe] catalytic site with one additional [4Fe-4S] cluster, HoxEFU contains an FMN cofactor in HoxF, as well as seven FeS clusters (three [2Fe-2S] clusters and four [4Fe-4S] clusters) (8). EPR and FTIR spectroscopies have been employed for biophysical characterization of S. 6803 HoxEFUYH, and have revealed some unusual characteristics compared to standard [NiFe] hydrogenases (15).

During purification HoxEFUYH is known to readily dissociate to form HoxEFU sub-complexes separate from the HoxYH hydrogenase sub-complex (16-19). This has raised the question of whether the HoxEFU sub-complex has a functionally distinct role in the cell (16,20). To date HoxEFU has been observed in vivo (16) as well as in vitro following isolation of the pentameric complex, exemplified by the observation that in thylakoid membrane imaging, ~23% of HoxF is found incorporated into the HoxEFU subcomplex rather than the complete HoxEFUYH complex (20).

The details on the fundamental biochemistry of S. 6803 HoxEFU, its biophysical properties, substrate preferences, reaction kinetics, and structural features, specifically the role of HoxE in mediating reactions with soluble electron carriers, are unresolved. HoxE in S. 6803 is known to be required for H₂ production by HOX coupled to both NADH and NADPH (7), and has been implicated in binding and electron transfer with Fdx and to also facilitate association of HOX with the thylakoid membrane (20). Investigations from Thiocapsa roseopersicina have further supported the role of HoxE in electron transfer (21). Thus, the HoxE subunit is required for several important functions of photosynthetic HOX complexes, though specific features that contribute to diaphorase activity are not completely known. The HOX from C. necator, in contrast, reversibly catalyzes the oxidation of NADH in the absence of HoxE, although it does include the non-homologous HoxI. Unlike HoxE, HoxI does not bind an FeS cluster (12,17) and is therefore thought not to play a role in electron transfer. The HOX complex from Hydrogenophilus thermoluteolus catalyzes H₂ oxidation coupled to NAD⁺ reduction, and has neither a HoxE nor HoxI subunit (22). Thus, how subunit compositions are linked to biochemical functions across HOX diversity and the role of individual subunits remain to be resolved.

To address questions about the relationship of structural and compositional properties of S. 6803 HOX to its biochemical function, the S. 6803 HoxEFU was isolated as an intact sub-complex and the biophysical properties, reactivity and structural composition were determined. The results demonstrate that HoxEFU cooperatively couples NAD(P)H oxidation and reduction (diaphorase activity) to the exchange of electrons with other redox carriers including Fdx’s and Fld, all in the absence of the HoxYH hydrogenase sub-complex. The biochemical and structural features identified here demonstrate that HoxEFU has the essential
biochemical properties to catalyze redox reactions with photosynthetic electron transfer components in a more nuanced and dynamic manner than previously established.

Results and Discussion

Initial expression and characterization of HoxEFU: Heterologous expression of HoxEFU in Escherichia coli was carried out based on the procedure previously described for HoxEFUYH in S. 6803 (15). The expression construct included a Strep-II tag on the C-terminus of HoxF and conserved ribosomal binding sites from the Hox operon in S. 6803. Affinity purification under anaerobic conditions yielded ~1.5 mg of HoxEFU per-liter of growth medium, and mass spectrometry was used to verify subunit composition (Fig. S1) and flavin incorporation. FMN incorporation was verified through UV-Vis, with a ratio of 0.7 FMN per HoxEFU.

EPR of HoxEFU with natural electron donor-acceptors: To determine whether HoxEFU can react with NAD(P)H and mediate electron transfer reactions via the FeS clusters, the effect of NAD(P)H on the reduction-oxidation state of the HoxEFU cofactors was determined by EPR. The anaerobically as-purified HoxEFU was EPR silent (Fig. 2, green spectrum) due to the lack of added reductant (such as NAD(P)H or sodium dithionite (DT)) during the purification process. To determine the spectrum of the reduced HoxEFU complex, the as-purified sample was reduced with DT ($E_m = -660$ mV vs NHE, pH 7 (23)). This resulted in a spectrum with an overall rhombic line-shape that accounted for $6.5 \pm 1.5$ spins mol$^{-1}$ (Fig. 2, blue spectrum), compared to the expected count of 7 spins mol$^{-1}$ for full incorporation of FeS clusters.

Reduction of HoxEFU by the physiological donors NAD(P)H yielded spectra similar to the DT-treated sample, with $5.3 \pm 1.5$ spins mol$^{-1}$, indicating near complete reduction (Fig. 2, black and red spectra), and confirming the ability of the pyridine nucleotides to react with FMN. The slightly lower spin concentration compared to the DT-treated sample likely results from one of the FeS cofactors not being fully reduced by NAD(P)H, and evidenced by a less intense spectral feature at $g = 2.0$ (see feature highlighted with an arrow in Fig. 2B compared to Fig. 2C and 2D) in NAD(P)H versus DT reduced HoxEFU. Interestingly, reduction of HoxEFU by NAD(P)H was more effective in reducing the accessory clusters ($\sim5$ spins mol$^{-1}$) compared to HoxEFUYH at 1.9 spins mol$^{-1}$ (15). This most likely is due to HoxEFUYH being under turnover conditions for $H_2$ production (unlike HoxEFU which cannot catalyze reduction of protons), which could prevent the observation of fully reduced FeS clusters under steady-state conditions.

All reduced samples showed strong intensity near the middle of the spectrum ($g = 1.93$) and broad features at the wings (most prevalent at low temperature). The broadening likely reflects magnetic coupling between the multiple reduced FeS clusters, consistent with their function in electron transfer for HoxEFU (15,24). Several features at the low and high field regions of the spectrum showed similarity to the FeS cluster signals reported for the HoxEFUYH pentameric complex (15), and the signals could be further resolved based on temperature and microwave power dependency (Fig. S3). This includes slower relaxing signals (optimal temperature 25K) at $g = 2.00, 1.94,$ and 1.90 and faster relaxing signals (optimal temperature 4K) at $g = 2.05, 1.98,$ and 1.88 which are consistent with [2Fe-2S] and [4Fe-4S] clusters, respectively. To further corroborate these assignments, EPR simulations were carried out on the DT-reduced HoxEFU sample (Fig. S3). Unlike the prior report of HoxEFUYH (15), the simulations did not readily converge when only two spin systems were included, however, the addition of a third system was able to account for the measured EPR signal. The simulations include two systems ($g = 2.16, 1.939, 1.929,$ and $g = 1.998, 1.945, 1.913$) attributable to [2Fe-2S] clusters based on the temperature-relaxation properties that were in close agreement to the $g$ values of the prior report, and one broad system ($g = 2.047, 1.927, 1.875$) that accounts for nearly half the spin density in the overall signal (Fig. S3). The latter can be assigned to a [4Fe-4S] cluster type signal based on the temperature relaxation properties, though the greater weight of this signal in the simulation indicates it may have overlapping contributions from additional clusters, or the greater weight may be a result of a different effect such as power saturation at low temperature. The simulations...
begin to distinguish the various FeS cluster components of the overall signal, although full deconvolution is difficult due to the rich FeS cluster content of the protein. It should also be noted that the FMN semiquinone is not directly observed under NAD(P)H or DT reduction, rather it may be in the fully reduced hydroquinone and EPR-silent form or obscured by overlapping signals in the g = 2 region.

Table 1. Reaction kinetics of S. 6803 HoxEFU.

| Reaction                        | $K_m$ (µM) | $K$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$·s$^{-1}$) | $V_{max}$ (µmol min$^{-1}$ mg$^{-1}$) |
|--------------------------------|------------|----------|---------------------|----------------------------------|-------------------------------------|
| NADH oxidation to MB$^{1}$ reduction | 39 ($K_m$ for NADH) | 4.25 | 1.1x10$^5$ | 2.5 |
| NADPH oxidation to MB reduction | 1003 ($K_m$ for NADPH) | 9.69 | 9.6x10$^4$ | 5.7 |
| NADH oxidation to Fld reduction | ND$^2$ | 0.005 | ND | 0.003 |
| NAD$^+$ + Fld$_{red}$ | ND | 0.003 | ND | 0.002 |
| NADP$^+$ + Fdx$_{1red}$ | 15.4 ($K'$ for Fdx$_{1red}$) | 0.63 | 4.1x10$^4$ | 0.37 |
| NADP$^+$ + Fdx$_{1red}$ | 59 ($K'$ for Fdx$_{1red}$) | 3.8 | 6.4x10$^4$ | 2.2 |

$^{1}$MB, methylene blue; $^{2}$ND, not determined.

Reaction kinetics of HoxEFU with NAD(P)H, ferredoxin and flavodoxin: The HoxEFUYH complex couples the oxidation of reduced electron carriers to the production of H$_2$. HoxEFU is required for the reactivity with electron carriers, however, the binding site interactions and kinetics are not well known. To determine the reaction kinetics and preferences of HoxEFU for different electron carriers, we measured the reactivity in coupled reactions with redox dyes. The oxidation of NAD(P)H by HoxEFU (Table 1) is a half-reaction of the NAD(P)H-to-H$_2$ reaction catalyzed by HoxEFUYH, with $k_{cat}/K_m$ values of 1.1x10$^5$ and 9.6x10$^4$ for NADH and NADPH, respectively. The $K_m$ value of HoxEFU for NADH of 39 µM is within the previously reported values of 12-83 µM for the intact HoxEFUYH complex (8,10), whereas the measured HoxEFU $K_m$ value for NADPH of 1 mM is ~10-fold greater than was previously reported for H$_2$ production by S. 6803 cell extracts (8). This may reflect differences in assay conditions, or differences in the binding and reactivity of HoxEFU for NADPH in the absence of HoxXYH. Previous reports on HOX activity measured H$_2$ evolution or uptake on cell-extracts (4,7,10,11), or by electrochemical methods of purified enzymes where the quantity of electroactive enzyme was not defined (6,14). As such it is not possible to directly compare specific rates obtained here on purified HoxEFU with those previously reported results.

The NAD(P)H measurements employed coupled reactions using the redox dye methylene blue (MB, $E_m$ = +11 mV vs NHE), rather than the natural substrates (i.e. Fdx). To assess kinetics of a physiologically relevant reaction, HoxEFU activity was measured for NAD(P)$^+$ reduction coupled to Fdx$_{1red}$ ($E_m$ = -412 mV vs NHE (25)) oxidation. Unlike the dye-based assays, a plot of the reaction velocity indicated cooperative kinetics, with kinetic fits for NAD$^+$ and Fdx$_{red}$ having Hill coefficients of 2.8 (Fig. S5, Table S3). The observation of cooperativity suggests that HoxEFU might assemble into oligomers, where binding of Fdx$_1$ or NAD$^+$ to one HoxEFU could induce long-range effects on binding of Fdx$_1$ or NAD$^+$ at additional HoxEFU binding sites (26). In contrast, NADP$^+$ did not exhibit cooperativity with Fdx$_{1red}$, and a higher $K_m$ of 59 µM for Fdx$_{1red}$ was observed. The $k_{cat}/K_m$ values for both NAD$^+$ and NADP$^+$ reduction were lower than for NAD(P)H oxidation (Table 1). The kinetic assays demonstrate a systematic preference for NAD(H) over NADP(H), a trend consistent with prior measurements of NAD(P)H-dependent H$_2$ production by HoxEFUYH. (Table S1) (8,10,11).

Fdx serves as an electron carrier in peripheral photosynthesis pathways, and was previously shown to stimulate H$_2$ production by HoxEFUYH in whole-cell extracts (4). To assess whether HoxEFU is able to react with Fld as a substrate, the reduction of NAD(P)$^+$ with reduced Fld was tested. The redox couples of Fld are ~ -433 and -240 mV vs NHE, for the fully reduced hydroquinone and semiquinone, respectively (25). Based on the relative thermodynamic favorability of the NADP$^+$/NAD(P)H redox couples ($E_m$ = -320 mV),
it seemed reasonable that HoxEFU could mediate Fld-based reduction of NAD\(^+\), as well as the reverse reaction. Indeed, HoxEFU coupled oxidation of Fld\(_{\text{red}}\) to the reduction of NAD\(^+\) at a lower \(k_{\text{cat}}\) of 0.003 s\(^{-1}\) compared to the Fdx1\(_{\text{red}}\) reaction (\(k_{\text{cat}}\) of 0.63 s\(^{-1}\)) (25). The formation of the Fld semiquinone species was observed concurrently with the formation of NADH. In the reverse direction, HoxEFU oxidized NADH and reduced Fld to the semiquinone state, with a \(k_{\text{cat}}\) of 0.005 s\(^{-1}\), after an initial lag period. In contrast to Fdx1, the one-electron reduction of Fld (\(E_m = -240\) mV vs NHE) from NAD(P)H oxidation (\(E_m = -320\) mV vs NHE) is thermodynamically more favorable. The \(k_{\text{cat}}\) of HoxEFU mediated reduction of NAD\(^+\) by oxidation of Fld\(_{\text{red}}\) was \(10^3\) s\(^{-1}\), ~100-fold lower than the reaction using Fdx1\(_{\text{red}}\). In cell extracts, reduced Fld stimulated higher \(\text{H}_2\) production rates than Fdx1 (with HoxEFUYH), with a lower apparent \(K_m\) than Fdx1 (4). Based on our kinetic results it is suggested there might be conformational and/or reactivity differences of HoxEFU compared to HoxEFUYH that may regulate the substrate preferences and/or reaction kinetics differently than for HoxEFUYH to favor Fdx over Fld.

**Reactivity with additional ferredoxins:** The ability of HoxEFU to reduce Fld suggests that other soluble redox carriers with similar midpoint potentials may also serve as redox partners. To test this, diaphorase activity was measured under standardized conditions for an additional four Fdx’s from \(S.\) 6803 (Table 2, sequence alignment in Fig. S2), using previously described gene numbering (27). These assays were performed using estimated \(V_{\text{max}}\) conditions of 50 \(\mu\)M Fdx and 2 mM NAD(H), though the \(k_{\text{obs}}\) values may not be equivalent to \(k_{\text{cat}}\).

Fdx2 (ssl138) has a midpoint potential of \(E_m = -243\) mV, (28) similar to the \(E_m\) of the Fld semiquinone. Notably, Fdx2 has a unique intracellular role compared to Fdx1 in mediating iron homeostasis and chlorophyll accumulation, and is conserved in photosynthetic microbes (27,29). While the rate of NADH oxidation to reduction of Fdx2\(_{\text{ox}}\) was low (\(k_{\text{obs}} = 0.02\) s\(^{-1}\)), it was 10-fold higher than with Fld\(_{\text{ox}}\). The reverse reaction of NAD\(^+\) reduction by Fdx2\(_{\text{red}}\) was not detected.

Fdx4 (ssl0150) is closely related to Fdx1, although they have different expression and interaction profiles (27). Analysis of the homologous Fdx from *Trichodesmium elongatus* revealed a midpoint potential of \(-440\) mV vs NHE (30), and has been shown to stimulate \(\text{H}_2\) production in \(S.\) 6803 HoxEFUYH (4). Thus, it was hypothesized that it would support NAD\(^+\) reduction by HoxEFU to a similar degree as Fdx1, and indeed this was found to be the case, with a \(k_{\text{obs}}\) of 0.81 s\(^{-1}\), slightly higher than that of Fdx1.

Fdx5 (ssl0148) is found on the same operon as Fdx4, and thus is similarly regulated. However, Fdx5 harbors a [2Fe-2S] cluster, is more closely related to bacterial than plant type Fdx, and is more distantly related to Fdx1 (27). Under the conditions we tested, HoxEFU showed no activity with Fdx5 for either NAD\(^+\) reduction or NADH oxidation. The lack of activity may be due to some factor present in the cell that was not captured in the *in vitro* assay, such as phosphorylation (31), or may be a result of inefficient binding and electron transfer between Fdx5 and HoxEFU.

An additional cyanobacterial Fdx which we deem Fdx11(ssl3044) was discovered via cyanobase, and putatively assigned as a [2Fe-2S] cluster Fdx of 10.8 kDa. Potential Fdx11 interaction partners predicted by the STRING database (32) include the photosynthetic components psbO and psaF. Fdx11 was found to be able to support the reduction of NAD\(^+\) to NADH by HoxEFU with a \(k_{\text{obs}}\) of 0.10 s\(^{-1}\), consistent with a midpoint potential below that of the NAD\(^+\)/NADH couple. This activity establishes a new reactivity pathway for HoxEFU which may involve photosynthetic electron transfer.

Collectively the results with the different Fdx’s demonstrate that HoxEFU has the capacity to react with a range of physiological electron carriers, and that the direction of the reaction is highly potential-dependent. Size, surface charge, and midpoint potential are all factors that may influence binding and reactivity. The relative contributions of these factors are difficult to determine given that to date the only Fdx from \(S.\) 6803 that has a solved crystal structure is Fdx1 (33). The number of Fdx’s that can react with HoxEFU implicate that HOX functions within a complex reactivity network and suggests the possibility that HoxEFU may react with other electron carriers such as other ferredoxins, cytochromes, or quinones. The diaphorase activity
of HoxEFU therefore might function independent of HoxYH and hydrogenase activity to exchange reducing equivalents among the carrier pools in cells. However, this requires further in vivo investigation.

| Table 2. Reactivity of S. 6803 HoxEFU with Fdxs. |
|------------------|------------------|------------------|------------------|
| Fdx   | NAD$^+$ reduction ($k_{obs}$, s$^{-1}$) | NADH oxidation ($k_{obs}$, s$^{-1}$) | $E_m$ (mV vs NHE) |
| Fdx1  | 0.63 | Not observed | -412 (25) |
| Fdx2  | Not observed | 0.02 | -246 (28) |
| Fdx4  | 0.81 | Not observed | -440 (30)$^2$ |
| Fdx5  | Not observed | Not observed | Not determined |
| Fdx11 | 0.10 | Not determined | Not determined |

$^2$Previously assigned as Fdx3 (4). $^2E_m$ value of the Fdx1 homologue from T. elongatus (30). $^3$Fdx11 was identified in this work via Cyanobase (34).

The activities measured here demonstrate that the HoxEFU sub-complex has substrate reactivities both complementary to and distinct from the intact HoxEFUYH complex for coupling redox reactions between NAD(P)$^+$H, and Fdx or Fld. The fact that HoxEFUYH is further able to couple Fdx, Fld or NAD(P)$^+$H to H$_2$ activation, implies that it may manage a single redox reaction cycle utilizing 3 redox substrates (4). This reactivity would be similar to the electron bifurcation reaction that has been observed for [FeFe]-hydrogenases from anerobic microbes (35,36). On the other hand, the NAD(P)$^+$H-Fdx/Fld linked reactivity of HoxEFU may be specific to the sub-complex to afford flexible channeling of electron flow among substrate pools. It has also been hypothesized that HoxEFU functions to reduce, and thereby reactivates the oxygen-inactivated [NiFe] site in HoxYH, a hypothesis that is not excluded by our results (6). The ability of HoxEFU to mediate an exchange of electrons between the NAD(P)$^+$H, Fdx and Fld pools are especially interesting in light of the recent discovery that the cyanobacterial photosynthetic complex I (NDH-1) exclusively accepts electrons from reduced ferredoxin (37).

*Equilibrium Binding Isotherms for NAD(P)H: In order to further evaluate the cooperativity observed by the Fdx1/NAD$^+$ kinetic assays, we performed fluorescence resonance energy transfer (FRET) studies on HoxEFU, in which the binding interactions of NADH to HoxEFU are monitored by the fluorescence emission. The equilibrium binding isotherm demonstrates that NADH binds cooperatively to HoxEFU, with a Hill coefficient of 1.9, and a $k_d = 32 \mu$M, close to the $K_m$ of NADH in the MB reduction reaction (Fig. S4A). We also examined the equilibrium binding isotherms of NAD$^+$ and NADP$^+$ to HoxEFU. Both pyridine nucleotides showed strong binding cooperativity, with Hill coefficients of 1.77 ± 0.07 and 1.70 ± 0.15 for NAD$^+$ and NADP$^+$, respectively, and $K_d$ values of 1350 ± 70 and 1950 ± 270 µM (Figure S3 B and C, Table S3). Thus, NADP$^+$ dissociates from HoxEFU more readily than NAD$^+$, consistent with activity measurements that show a stronger preference for NAD$^+$ over NADP$^+$. The $K_d$ values also reflect pyridine nucleotide concentrations that are likely well above the concentration of ~80 nmol per-g of fresh cell weight previously reported for S. 6803 (38). Based on the cooperativity that was observed, only a fraction of HoxEFU will bind with NAD(P)$^+$ at concentrations below ~500 µM, with a steep increase in bound pyridine nucleotide to ~2 mM. The binding kinetics agree well with the reaction kinetics results, and support a function of HoxEFU sub-complex in catalytic oxidation of NAD(P)H coupled to reduction of soluble carriers, rather than the Fdx-dependent reduction of NAD(P)$^+$. Although oxidation of NAD(P)H was observed using MB as an artificial electron acceptor, no oxidation was observed when MB was replaced with Fdx$_{ox}$. This is likely a result of unfavorable thermodynamics of reducing a more negative potential Fdx (Fdx1, $E_m = -412$ mV vs NHE) by the more positive potential of NAD(P)H ($E_m = -320$ mV) (Fig. 3). Under different intracellular conditions when there is a high concentration of NAD(P)$^+$ relative to NADPH, such as in cases of dark-to-light transition (39,40), or under low-light and nitrogen deprivation (41), HoxEFU may catalyze Fdx$_{red}$-dependent reduction of pyridine.
nucleotides in a reaction similar to that catalyzed by FNR. The $K_d$ results suggest that this is not a primary function of HoxEFU, though it remains a possibility that the addition of the HoxYH subcomplex could modify reactivity.

The demonstration that Fdx1 can donate electrons to HoxEFU supports the capacity of HoxEFUYH to mediate Fdx1-dependent evolution of $H_2$ (4), perhaps without a requirement for NAD(P)H oxidation. An additional scenario is that HoxEFUYH is capable of a coupled reaction such as electron bifurcation, which is suggested by the relative midpoint potentials of HoxEFUYH’s redox cofactors. Indeed, the low $k_{cat}/K_m$ value of $10^4$ identified for HoxEFU in the Fdx1-dependent reduction of NADP+ may be an outcome of a short-circuited bifurcation reaction in the absence of HoxYH. To our knowledge there are no reports in the literature of assays that have measured HoxEFUYH bifurcation.

It is an intriguing observation that the NADP+-based kinetics demonstrate a standard Michaelis-Menten type hyperbolic curve, while the equilibrium binding isotherms show sigmoidal Hill-type behavior. This suggests the possibility that binding is indeed cooperative, but that the NADP+ reduction reaction is only observed once the binding sites are saturated (42,43). Prior work on glucokinase has shown that addition of a second substrate changes the observed kinetics from sigmoidal to hyperbolic, in a sense, masking cooperativity (44,45). Kinetics are known to differ from equilibrium binding particularly in situations such as when substrate binding is slow or rate limiting (46). Furthermore, the observation of hyperbolic kinetics does not exclude the reaction from involving a cooperative mechanism (47).

Identification of the Fdx1 binding site by Mass Spectrometry: The binding site of Fdx1 on HOX has been implicated to involve the HoxE subunit, though the biochemical evidence for a specific binding interaction is lacking (4,20). The binding interaction of Fdx1 with HOX has important contributions to the control of reaction cooperativity and the electron flow within HOX during turnover. To address this, chemical cross-linking experiments between purified Fdx1 and purified HOX, along with LC-MS/MS, were undertaken to identify potential binding sites of Fdx1 to HoxEFU (Table S4). The identified cross-links place Fdx1 in a cleft between HoxE, HoxF, and HoxU (Fig. 4). Additional Fdx1-HoxEFU cross-links that were identified, may provide alternative binding configurations or be an outcome of allosteric or oligomeric (monomer or dimer) state changes in HoxEFU.

Our cross-linking mass spectrometry results support the role of Fdx1 in binding simultaneously to HoxE, HoxF, and HoxU, allowing electron transfer from Fdx1 to the [2Fe-2S] cluster in either HoxE or HoxF, with the branched arrangement of FeS clusters in HoxU allowing for multiple possible electron transfer routes between the active site and other interaction partners. It is important to note that HoxU shows a strong structural similarity to the N-terminal part of [FeFe]-hydrogenases which holds its four accessory FeS clusters. Since [FeFe]-hydrogenases are well known Fdx-oxidizing enzymes one of the FeS clusters of HoxU might be an electron recipient in the HoxEFU module as well as in HoxEFUYH. The most closely related structural model to S. 6803 HOX is from Hydrogenophilus thermoluteolus, it lacks HoxE and has a HoxF with significant sequence differences to S. 6803 HoxF. Thus, the S. 6803 HoxEFU-Fdx1 model that has been obtained here represents a significant advance in understanding the binding site of Fdx1 to S. 6803 HoxEFU. Prior biochemical results on HoxEFUYH from S. 6803 have shown that Fdx1 is a competent electron donor for catalyzing $H_2$ production, which may involve Fdx1 binding to the site identified here for HoxEFU, and/or additional binding sites (4).

Conclusions

Collectively, our results show that HoxEFU is capable of functioning in vitro, independent of HoxYH, to couple redox reactions between NAD(P)H, Fdx1, Fdx2, Fdx4, Fdx11, and Fld (Fig. 3). The variability in the efficiencies of different ferredoxins to exchange electrons with NAD(P)H via HoxEFU underpins the idea that the high number of ferredoxins in Synechocystis supports a robust network of redox regulation. It remains to be shown if the ability of the diaphorase to shuttle electrons between the NAD(P)H, Fdx and Fld pools is of physiological importance in vivo. We have
identified cooperative binding kinetics of HoxEFU, which are important for reactivity with physiological electron donors. Cross-linking mass spectrometry provides a structural model for how Fdx1 interacts with HoxEFU. The addition of HoxYH may further tune HoxEFU activity by adding electron transfer pathways and possibly modifying the oligomeric state of the HOX complex. Catalytic rates of pyridine nucleotide reduction by HoxEFU versus HoxEFUYH suggest that substrate reactivity changes in the presence of HoxYH or other interaction partners. While many of the functions of HoxEFU identified here may be conserved among other HOXs, changes to subunit composition such as in H. thermoluteolus, which lacks HoxE (22), and C. necator, which replaces HoxE with the unrelated HoxI (12), could alter both reactivity and higher-order structure. Our results will enable further efforts to understand the structural and biophysical mechanisms related to biological electron transfer, and the role of HoxEFU in photosynthetic processes.

**Experimental Procedures**

**Protein expression and purification:** HoxEFU expression was similar to that previously reported for S. 6803 HoxEFUYH (15). The Hox operon was modified to add a Strep-II tag with a serine-alanine linker on the C-terminal end of HoxF. The protein encoding genes of unknown function were removed from the gene construct, but the intergenic regions encoding for ribosomal binding sites were conserved. The hoxE, hoxF, and hoxU gene sequences were optimized for expression in Escherichia coli using GenScipt’s proprietary software. Genscript synthesized the gene and cloned it into the pET21 vector.

The pET21HoxEF*U vector was transformed into ΔiscR, kan² BL21 competent cells. Five fresh colonies were used to inoculate a 150 mL TB media overnight culture, which was grown at 37°C and 225 rpm. After 16 h, 3 mL of the overnight was inoculated into 1L of pre-warmed TB media and grown to O.D. 600 nm of 0.4. To induce protein expression the cultures were treated with 1 mM IPTG and supplemented with ferric ammonium citrate (4 mM final), cysteine (2 mM final), sodium fumarate (25 mM final) and flavin mononucleotide (10 µM final), and sparged with Argon overnight at room temperature. All subsequent treatments were strictly anaerobic. Cells were harvested by centrifugation at 6037 RCF for 5’, resuspended in buffer, and frozen at -80 °C.

For purification, EDTA-free protease tablets, lysozyme, and DNase were added to thawed cell pellets, which were then lysed by passage through a microfluidizer 10-12 times. The lysate was then centrifuged at 149,000 RFC for 1 hr, and the clarified lysate was applied directly to a Strep-XTHC column. Elution was performed with 10 mM biotin, with a typical yield of purified protein at 1.5 mg L⁻¹ of culture.

HoxEFU protein identification from both in gel and in solution digestion were performed according to standard protocols recommended by the manufacturer using a trypsin (Promega) protease:complex ratio of 1:50-1:100 overnight and for three hours, respectively. Proteins were identified as described (48) using a maXis Impact UHR-QTOF instrument (Bruker Daltonics) interfaced with a Dionex 3000 nano-uHPLC (Thermo-Fisher) followed by data analysis in Peptide Shaker v.1.13.6 (49). Intact protein analysis was performed as described previously using a Bruker microO-TOF mass spectrometer (Bruker Daltonics) coupled to a 1290 ultrahigh pressure (UPLC) series chromatography stack (Agilent Technologies) (50,51).

Fdx1 expression was performed by transformation into BL21 competent cells. 5-10 colonies were used to inoculate a 150 mL LB overnight culture and shaken at 225 rpm, 37 °C. This culture was then used to inoculate 1 L of LB media, and the cells were grown to O.D. 600 nm of 0.6, and expression was induced with 1 mM IPTG and supplemented with ferric ammonium citrate (4 mM final) and cysteine (2 mM final). After continued growth for four hours cells were harvested by centrifugation at 6037 RCF for 5’, resuspended in buffer, and frozen at -80 °C. The thawed cell pellet was sparged with argon for 10’, and all subsequent steps were handled anaerobically. Lysis and purification were carried out as with the HoxEFU, yielding ~4 mg L⁻¹ of culture.

**Fdx2, Fdx4, Fdx5 and Fdx11 expression and purification:** Fdx2, Fdx4, Fdx5 and Fdx11 from Synechocystis sp. PCC 6803 (sll1382, slr0150,
slr0148 and ssl3044, respectively) were cloned into a modified version of the pRSETA vector (Life Technologies, USA; (52,53) that lead to the expression of a FDX-TEVcs-GST-His fusion protein with a linker sequence (54) between the FDX and the GST-His tandem affinity tag. E. coli KRX cells (Promega, Germany) were used to over-express the protein over-night in LB at 10 °C after induction at OD$_{600}$ of 0.6 with IPTG and Ferric ammonium citrate at final concentrations of 1 mM and 0.05 % (w/v), respectively. Cells were harvested, resuspended in lysis buffer (50 mM NaPO$_4$, pH=7.0, 250 mM NaCl) and broken by sonication (Sonopuls (Bandelin, Germany); Sontrode MS73; 8 repeats of 20 seconds on (70% cycle, 70% power) and 20 seconds off). The supernatant obtained after ultracentrifugation was incubated for 1 hour at 4°C with TALON Cobalt affinity chromatography resin (Takara, Germany). Following the incubation period, the resin was washed with 20 column volumes (CV) of lysis buffer. Protein elution was performed with 2 CV of elution buffer (50 mM NaPO$_4$, pH=7.0, 250 mM NaCl, 500 mM imidazole) and the eluted proteins were dialyzed over-night in 25 mM Tris pH, 50 mM NaCl in the presence of 20 mg TEV-His (His-tag purified from pRK193 (Addgene, USA) (55)). The following day the protein was incubated with TALON Cobalt affinity chromatography resin. The flow-through was concentrated and loaded onto a HiLoad™ 26/60 Superdex™ 75 prep grade (GE Healthcare, Germany) following the purification method developed by Peden et al. (56).

Flavodoxin expression, purification, and biochemical assays. Flavodoxin (isiB) from Synechococcus sp. PCC 7002 was recombinantly expressed in E. coli BL21 (DE3) as described previously (57). Cells were broken by French Press and spun at 35k rpm for 1 hour at 4C. The resulting supernatant was loaded to a DE-52 anion exchange column equilibrated with 50 mM Tris pH 8, then washed with a gradient of 50-100 mM NaCl, and finally eluted with 500 mM NaCl. Colored fractions were collected, washed with 50 mM Tris pH 8, 20 mM NaCl and concentrated by ultrafiltration. An extinction coefficient of 9500 M$^{-1}$ cm$^{-1}$ at 467 nm was used to determine flavodoxin concentration.

Biochemical assays were performed using flavodoxin in a 115-fold excess to HoxEFU, in the presence of 2 mM NAD(P)H. Reduction of flavodoxin by HoxEFU was monitored by following the decrease of absorbance at 467 nm and concurrent increase at 580 nm. To assay activity in the reverse direction, flavodoxin was reduced with 100-fold molar excess of dithionite and thoroughly buffer exchanged. Oxidation of the fully reduced flavodoxin by HoxEFU was measured by the increase at 580 nm. Assays were initiated by the addition of NAD$^+$ or NADH, and the reaction was monitored at 340 nm for the NADH signal, and 467 and 580 nm for the oxidized and semiquinone species of flavodoxin, respectively.

Biochemical assays: Kinetics were measured using a Cary4000 UV-Vis spectrometer in kinetics mode. For pyridine oxidation the reaction was initiated by the addition of Methylene Blue and the reaction monitored at 666 nm. For pyridine reduction, reduced ferredoxin was added, and the pyridine signal at 340 nm was monitored. Curves were fit in OriginPro 2019. To prepare the reduced ferredoxin, the ferredoxin was treated with sodium dithionite (DT) in ~100-fold molar excess, and buffer-exchanged repeatedly via centrifugal filters. Removal of dithionite was verified by Uv-Vis at 316 nm. Activity measurements of Fdxs 2, 4, 5, and 11 were carried out using 0.3 µM HoxEFU, 50 µM Fdx, and 2mM NAD(H).

NAD(P)H equilibrium binding kinetics were performed using FRET similar to previously described assays (58,59). Fluorescence data was collected at an excitation wavelength of 285 nm, emission from 300-500 nm, with slit widths of 8 nm (Fluorolog 3, Horiba). Intrinsic protein fluorescence emission at 348 nm was used to calculate the binding isotherm.

EPR spectroscopy: CW X-band EPR was carried out on a Bruker Elexys E-500 spectrometer equipped with a helium cryostat and an SHQ resonator in conjunction with a MercuryITC temperature controller. Spectra were collected at a frequency of 9.38 GHz, power and temperature as noted in the figures, modulation frequency of 100 kHz, and modulation amplitude of 10 GHz. Data was baseline corrected as needed with a user-defined function in OriginPro 2019. Simulations were carried out using the “pepper” function in EasySpin 5.2.25 (60). Spin quantitation was
performed using the double-integrated EPR spectra and referenced to copper triethylamine samples of known concentration (75 and 100 µM) measured under the same conditions.

Cross-linking and protein modeling: Protein-protein interactions within the HoxEFU complex and HoxEFU trimer with Fdx1 were examined using chemical cross-linking (61). Briefly, 1.5 µM HoxEFU was chemically cross-linked with 1 mM bis(sulfosuccinimidyl)suberate (BS3) (Thermo-Fisher) in 50 mM HEPES/150 mM NaCl buffer pH 7.2. The HoxEFU-Fdx1 the complex was established by mixing HoxEFU:Fdx1 in 1:6 ratio and incubated at room temperature for 2 min. Then the complex was exposed to BS3 for 15, 30, or 60 min at room temperature. All cross-linking reactions were quenched by adding TrisHCl, pH 8 to a final concentration of 120 mM. After fifteen minutes of incubation, the resulting mixtures were separated by SDS PAGE (4-20% linear gradient mini gel, Bio-Rad) and stained with Coomassie Brilliant Blue (Thermo-Fisher). Next, the entire gel lanes between 15-100kDa (according to the broad range marker migration profile, Bio-Rad) were digested with trypsin and the generated peptides were analyzed as described before (48). Cross-linked species were identified using Spectrum Identification Machine (SIM, v.1.2.2.2) (62) and MetaMorpheus (version 0.0.301) as described previously (63).

HoxEFU protein homology models were generated by Phyre2 (64) and energy-minimized models were docked using ClusPro2 with restrictions derived from cross-linking experiments (65-68). Ligand binding site prediction was run in 3DLigandSite (69). The FMN and iron sulfur cluster co-factors were docked using PatchDock (70,71) for individual subunits, compared to prediction based on 3DLigandSite and eventually added as rigid bodies to the final HoxEFU-Fdx1 complex model. Molecular graphics were created using PyMOL (72).

Data Availability
All data are contained within the manuscript.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. Scheme of the multimeric HoxEFUYH bidirectional hydrogenase from S. 6803. The complex is comprised of HoxEFU diaphorase (blue) and HoxYH [NiFe]-hydrogenase (orange) subunits containing a flavin (FMN) cofactor and [NiFe]-active site, respectively, along with multiple iron-sulfur clusters (sphere representation).
Figure 2. CW X-band EPR of HoxEFU prepared under different reduction-oxidation conditions. (A) As-purified. (B) Dark blue, reduced with 20 mM sodium dithionite, light blue, simulated spectra with the individual spin systems (Sys) specified above. (C) Reduced with 10 mM NADH. (D) Reduced with 10 mM NADPH. For all samples, HoxEFU (50 µM) was prepared in Tris buffer at pH 8.3. The EPR spectra were collected at 15K and 1 mW microwave power.
Figure 3. Model of *S. 6803* HoxEFU reactivity. HoxEFU catalyzes diaphorase reactions either accepting electrons from lower-potential donors such as Fdx$_{\text{red}}$ or Fld$_{\text{red}}$ (dotted red box) or donating electrons to higher-potential acceptors such as Fdx$_{2\text{ox}}$ or Fld$_{\text{ox}}$ (dotted blue box).
Figure 4. HoxEFU–Fdx1 binding model based on cross-linking-mass spectrometry and homology modeling of HoxE, HoxF, and HoxU subunits. Fdx1 (brown) is modeled to bind in a cleft between the HoxE (purple) and HoxU (gold) subunits. The iron-sulfur and flavin (FMN) content of the individual subunits are depicted as spheres.
The structure and reactivity of the HoxEFU complex from the cyanobacterium *Synechocystis* sp. PCC 6803

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