Dinitrogenase-Driven Photobiological Hydrogen Production Combats Oxidative Stress in Cyanothece sp. Strain ATCC 51142

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ABSTRACT
Photobiologically synthesized hydrogen (H2) gas is carbon neutral to produce and clean to combust, making it an ideal biofuel. Cyanothece sp. strain ATCC 51142 is a cyanobacterium capable of performing simultaneous oxygenic photosynthesis and H2 production, a highly perplexing phenomenon because H2 evolving enzymes are O2 sensitive. We employed a system-level in vivo chemoproteomic profiling approach to explore the cellular dynamics of protein thiol redox and how thiol redox mediates the function of the dinitrogenase enzyme complex in situ. Here, we demonstrate that high levels of hydrogen synthesis can be induced as a protection mechanism against oxidative stress via the dinitrogenase enzyme complex in Cyanothece sp. strain ATCC 51142. This is a previously unknown feature of cyanobacterial dinitrogenase, and we anticipate that it may represent a strategy to exploit cyanobacteria for efficient and scalable hydrogen production. We utilized a chemoproteomic approach to capture the in situ dynamics of reductant partitioning within the cell, revealing proteins and reactive thiols that may be involved in redox sensing and signaling. Additionally, this method is widely applicable across biological systems to achieve a greater understanding of how cells navigate their environment and how redox chemistry can be utilized to alter metabolism and achieve homeostasis.

Advancements in alternative fuel development are essential to alleviating the energy demands of our expanding global population. Hydrogen (H2) gas is a promising carbon-free fuel that can be enzymatically produced by photosynthetic microbes using electrons derived from photosystem II (PSII)-driven water photolysis (1–3). The dinitrotropic unicellular cyanobacterium Cyanothece sp. strain ATCC 51142 (referred to here as 51142) utilizes a dinitrogenase complex (NiFHDK) to fix N2 from the atmosphere, with H2 as a by-product (4, 5). In the absence of N2, NiFHDK can serve as a strict hydrogenase and reduce protons to H2 (Fig. 1) (1). High rates of NiFHDK-mediated H2 production can be achieved for long periods of time by manipulating the environment of Cyanothece sp. 51142 (4, 5), but our understanding of the underlying physiological dynamics that facilitate H2 production is limited.

Cyanobacteria require robust mechanisms for managing redox homeostasis and optimized metabolic rates. While the Calvin-Benson cycle is a major sink for reductant under nonlimiting CO2, there are alternative electron acceptors that act outside the photosynthetic linear electron flow (LEF) to prevent quinone pool hyperreduction. These pathways are important to the cell’s ability to protect itself from damage associated with redox imbalance (6–9). The signaling mechanisms that instigate alternative electron flow (AEF) processes to protect the cell from redox imbalance are ill defined (10). We hypothesize that redox-driven processes may provide the signal for multiple AEFs, allowing for flexibility of electron flow in the cell’s pursuit to regulate redox homeostasis.

Here, we demonstrate NiFHDK-driven H2 production as a potentially important AEF strategy employed by Cyanothece sp. 51142 to achieve redox balance, and we illuminate the broad role that redox sensing and signaling may play in a cell experiencing carbon and/or nitrogen limitation that can alter metabolic output.

To characterize the system-level events occurring during sustained photosynthetically supported H2 production, Cyanothece sp. 51142 cells held under an Ar atmosphere were transitioned from N-limited chemostat growth to a N-depleted state and incubated with or without CO2. The cell dynamics were interrogated via a chemical probe-based chemoproteomic technique (11–13) to quantify which specific protein redox dynamics in vivo were correlated with H2 production. Along with identifying the pro-
CM-H$_2$DCFDA assay for ROS. The oxidative stress indicator chloromethyl-2',7'-dichlorofluorescin diacetate (CM-H$_2$DCFDA; Cayman Chemicals) was used to compare ROS levels between time course samplings. Cells were collected from the photobioreactor during each sampled time point, and three aliquots (1 ml) were each treated with 10 mM CM-H$_2$DCFDA freshly dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to a final concentration of 10 μM. Additionally, three aliquots (1 ml) of cells were treated with 1 μl of DMSO as negative controls for the assay. Cells were transferred in three aliquots of 300 μl to opaque black 96-well plates (Nunc) per sample. Samples were incubated in the dark with intermittent shaking for 30 min at room temperature. Fluorescence was measured at 525 nm after excitation at 488 nm on a SpectraMAX Gemini XPS microplate reader. Relative fluorescent unit (RFU) values for each sampling were averaged, and error bars are included in Fig. 2.

In vivo labeling with protein redox chemical probes. Samples were concentrated by vacuum filtration on prewetted 0.45-μm Millipore nylon membranes. Nylon membranes with cells were transferred to 60-ml tissue culture dishes containing 2 ml of medium, 60 μM N-ethylmaleimide reductase probe (Mal-RP), and 60 μM iodoacetamide reductase probe (IAM-RP). Mal-RP and IAM-RP are cysteine thiol redox-reactive chemical probes, and when used together, as in this study, are referred to as IM-RPs (Fig. 3) (11, 12). Samples were incubated at 30°C in the dark for 60 min. Cells were then transferred to 2-ml conical tubes and centrifuged at 4°C and 4,000 × g for 2 min. Supernatant was removed, and cells were suspended with 1× phosphate-buffered saline (PBS) and centrifuged at 4°C and 4,000 × g for 2 min. This last washing step was repeated twice to remove unreacted probe, and the resulting cell pellets were flash-frozen with liquid nitrogen and stored at −80°C.

In vivo redox probe-labeled protein sample preparation. Cells were lysed by two rounds of bead beating using 0.1-mm glass beads and a Bullet blender (setting 8 of 10) for 4 min at 4°C. Lysate protein concentrations were measured using the bicinconinic acid (BCA) protein assay (Pierce), and each sample was adjusted to 360 μg with 1× PBS for normalization prior to subsequent preparation steps. Copper-catalyzed azide/alkyne cycloaddition “click chemistry” was performed as previously described (11) using a Tobacco etch virus (TEV) protease-cleavable azido-biotin tag (TEV-biotin-N$_3$), which includes an amino acid sequence recognized and cleaved by TEV protease. TEV-biotin-N$_3$ (36 μM), tris(2carboxyethyl)phosphine (TCEP) (2.5 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)m]amine (TBTFA) (250 μM) solubilized in a 4:1 tert-butanol–DMSO mixture, and CuSO$_4$ (50 μM) were added to each sample and the samples were incubated at 24°C for 90 min, thereby connecting probe-labeled samples to TEV-biotin. Samples were then processed for streptavidin affinity purification, trypsin cleavage, and TEV protease cleavage, as previously described (11), with the exception that only 260 μg of protein was enriched on 50 μl of streptavidin agarose resin slurry.

Global abundance proteomic sample preparation. From each sampling time point, two replicates of Cyanothece sp. 51142 whole-cell lysate (100 μg) were denatured and reduced by adding urea (8 M) and dithiothreitol (10 mM). Samples were incubated at 60°C for 30 min and then diluted 8-fold with NH$_4$HCO$_3$ (100 mM [pH 8.4]) to reduce salt concentration. CaCl$_2$ (1 mM) was added to the diluted samples, and proteins were digested using sequencing grade trypsin (Promega) at a ratio of 1 unit of trypsin per 50 units of protein for 3 h at 37°C. Digested samples were desalted using C$_{18}$ SPE columns (Supelco). Sample volumes were reduced by vacuum centrifugation and peptide concentrations determined. An equivalent amount of peptides from each sample/replicate was evaluated by liquid chromatography-mass spectrometry (LC-MS).

Redox probe-labeled protein and global abundance LC-MS analysis. Probe-labeled and no-probe control samples for in vivo experiments (see Data Set S1 in the supplemental material) and the global proteomic sample (see Data Set S3 in the supplemental material) for LC-MS analysis were analyzed using a Velos Orbitrap (Thermo Fisher Scientific) MS interfaced with a reverse-phase high-performance liquid chromatography (HPLC)
system for peptide separation (LC-MS), as previously described (11, 14). Data were acquired for 100 min, beginning 65 min after sample injection (15 min into gradient). Velos Orbitrap spectra were collected from \( m/z \) 400 to 2,000 at a resolution of 100,000 followed by data-dependent ion trap generation of tandem MS (MS/MS) spectra of the six most abundant ions using 35% collision energy. A dynamic exclusion time of 30 s was used to discriminate against previously analyzed ions.

We employed the accurate mass and time (AMT) tag approach for data analysis. The AMT tag approach utilizes tandem mass spectrometry to generate a reference peptide database (AMT tag database) of observed peptides, their associated theoretical masses, and LC elution times (normalized) (15). This database is utilized to assign peptide sequences to ion current (relative abundance) information of peptides measured using high-resolution high-mass-measurement-accuracy mass spectrometry. Generated MS/MS spectra were searched using the publicly available *Cyanothece* sp. 51142 translated genome sequence and then rescored using the MS-GF+ approach (16). Identified peptides of \( \geq 6 \) amino acids in length having an MS-GF score of \( \leq 1 \times 10^{-10} \), which corresponds to an estimated false-discovery rate (FDR) of \(< 1\%\) at the peptide level, were used to generate an AMT tag database. This database includes LC-MS measurements from *in vivo* probe-labeled samples and the global proteomic analyses. Measured arbitrary abundance for a particular peptide was determined by integrating the area under each LC-MS peak for the detected feature matching to that peptide. Matched features from each Velos Orbitrap data set were then filtered on an FDR of less than or equal to 1%; the FDR associated with the AMT tag is calculated using Statistical Tools for AMT.

**FIG 2** H\(_2\), dissolved O\(_2\), ROS, and protein redox profiles display intriguing and dynamic profiles that provide insight into the physiology of *Cyanothece* sp. 51142 during NifHDK driven H\(_2\) production. (A and B) Concentrations (in micromolar) of net H\(_2\) and O\(_2\) production and general ROS levels. (A) Sparged with pure Ar. (B) Continuous sparging with Ar and 1.3% CO\(_2\). Time zero indicates onset of NH\(_4^+\) depletion and absence of medium addition (dilution rate = 0). Times prior to \( t \) of 0 represent NH\(_4^+\)-limited chemostat steady-states. ROS, measured using CM-H\(_2\)DCFDA assay during specific time points throughout experiment and expressed as relative fluorescent units (RFU). (C and D) IM-RP-labeled proteins (scaled log\(_2\) abundances of the averages of three biological replicates) under both culture conditions. Each row represents a single protein, while each column is the average of three biological replicates. The color scheme of the heatmap is as follows: dark blue indicates high levels of reduction, yellow indicates high levels of oxidation, and gray indicates protein thiols were oxidized to a point in which any probe labeling is outside the limit of detection. (C) Four hundred fifteen proteins were probe labeled in the culture sparged with pure Ar. (D) Two hundred seventy-three proteins were labeled in the culture sparged with Ar plus 1.3% CO\(_2\). The list of proteins and AMT values can be found in Data Set S1 in the supplemental material.
state perturbation (these conditions, the metabolic dynamics that ensued upon steady-state perturbation (r = 0, Fig. 2A to D) showed strong evidence of cellular redox imbalance in attempt to recalibrate energy metabolism in response to N starvation (Fig. 2C and D). Upon N depletion, the H₂ production rate rose immediately, indicating that NifHDK was actively redirecting electrons toward proton reduction, although maximum and average concentrations of H₂ were ~25% less under CO₂-depleted conditions (Fig. 2A and B). The rates of oxygenic photosynthesis decreased substantially upon nutrient perturbation, as evidenced from decreased O₂ concentrations, which are potentially impacted by respiration and formation of ROS (21). Standing O₂ and H₂ concentrations, held during constant in-gas sparging of Ar, indicate net-positive rates of photosynthesis occurring concurrently with NifHDK-mediated H₂ production (Fig. 2A). Photosynthesis and H₂ production acted inversely over time, giving the first clue that NifHDK-driven H₂ production may play a role in protecting PSII from photoinhibition. Although not measured here, Melnicki et al. grew *Cyanothece* sp. 51142 under the same controlled growth conditions and determined that the cells lacked prominent photoinactivation, had stable chlorophyll levels, and maintained robust electron transport capacity (4).

H₂ production balances redox during oxidative stress. In cyanobacteria, if photon capture exceeds electron carrier and electron sink capacities, cellular stress ensues (22). An imbalance between excitation and electron capture and transfer results in electron accumulation that can reduce O₂, a by-product of water photoysis, and yield various forms of ROS, including O₂⁻, O₂(−) or H₂O₂ (23, 24). Under nutrient limitation, cyanobacteria have increased susceptibility to elevated ROS formation due to decreased availability of external (CO₂ and N₂) and biological (enzymes, NADP⁺, and plastoquinone [PQ]) electron acceptors (25, 26).

Intracellular ROS levels are temporally dynamic as *Cyanothece* sp. 51142 adjusts metabolically to N starvation and rebalances the redox state. This effect was measured in vivo by a broad-spectrum ROS-reactive dye (chloromethyl 2',7'-dichlorodihydrofluorescein diacetate [CM-H₂DCFDA]) (Fig. 2A and B). In CO₂-replete cultures, ROS levels rose gradually but peaked at 15 h, when H₂ concentrations were maximal (Fig. 2B). After this time point, H₂ and ROS levels fell but increased later in the sampled time course. These dynamics are potentially due to the rate-limiting features of an N-depleted condition, when nongrowing cells attempt to obtain appropriate C/N ratios (27). Once this metabolically active stationary phase occurs, the electron acceptor capacity decreases, thereby increasing potential for ROS production. After 21 h, the cells likely sense an imbalanced redox environment and reinit-
gate H₂ evolution. Without CO₂ available as a primary electron acceptor, photosynthetically produced electrons accumulate early in the time course; thus, ROS reaches the highest measured concentration by the first sampling at 3 h (Fig. 2A). These cells continue to evolve H₂ and subsequently ROS decreased to nearly N-limited steady-state levels by 18 h, when H₂ levels were maximal. These data clearly show an important correlation between H₂ production and ROS formation.

In vivo identification of redox-sensitive proteins. Cells use complex and elegant protein networks of redox-sensitive proteins to adjust metabolism and provide antioxidant protection in response to their environment (28). Characterizing Cyanothecae sp. 51142 protein redox dynamics during H₂ production provided a snapshot into the cells’ response to their environment and active reductant allocation (11–13). This was achieved by a chemoproteomic approach utilized to characterize in vivo redox dynamics with cysteine thiol redox-reactive chemical probes (IM-RPs) (Fig. 3). The IM-RPs include N-ethylmaleimide (Mal-RP) and iodoacetamide (IAM-RP) derivatives that cross cell membranes and covalently bind to reduced cysteine thiols directly in live cells, thereby yielding readout of protein redox dynamics under the physiological conditions of the experiment. Probe-labeled proteins are then characterized and quantified by LC-MS-based proteomic analyses (11–13). IM-RPs were applied to live cells over the time course at points corresponding to differing H₂ production inflections, and a tandem cleavage technique to characterize cysteine sites of probe labeling was utilized to gain insight into redox-sensitive thiols for labeled proteins (11, 29). Lists of the resulting probe-labeled proteins and sites of cysteine labeling can be found in Data Sets S1 and S2 in the supplemental material.

Probe-labeled protein profiles were dynamic and displayed intriguing patterns (Fig. 2C and D; see also Data Set S1 in the supplemental material for full quantitative results). Under the CO₂-depleted condition, proteins were more broadly reduced across the time course experiment, resulting in 415 redox-sensitive probe-labeled proteins; 159 of these proteins were differentially labeled compared to the steady-state samples. In contrast, only 273 proteins were labeled across the same time period under the CO₂-replete profile, 89 of which were differentially labeled compared to the steady-state precondition (see Fig. S1 and Data Set S1 in the supplemental material). The difference in the number of proteins undergoing redox events under the two conditions is attributable to CO₂ acting as a primary acceptor of photosynthetically derived electrons, resulting in fewer reduced proteins.

The probe-labeled protein data under both conditions revealed a general trend; initially, protein reduction and ROS levels increased, but as H₂ levels rose, proteins became more oxidized (Fig. 2C and D). This is likely driven by the depletion of reductant pools by NiFHDk-mediated H₂, which helps to oxidize the intracellular environment. As H₂ levels declined, protein probe labeling increased, indicative of a stronger reducing environment. The exception occurred at the 21-h point under the CO₂-replete condition. As H₂ levels declined, ROS increased dramatically, and proteins were the most oxidized at this time point, indicating a potential hysteresis effect from oxidative damage (Fig. 2D). In contrast to the general trend of protein redox and H₂ production, NiFHDk was increasingly reduced during elevated H₂ production and more oxidized when H₂ levels declined (Fig. 4). Additionally, we identified residue C218 as a site of labeling of NiF and believe it may be involved in sensing the redox environment.

These collective results support a relationship between NiFHDk-mediated H₂ production and ROS alleviation, highlighting the essential function of cyanobacterial NiFHDk as a sink of reductant to mitigate ROS stress. This concept is in accordance with a study that found that the heterocyst-forming diazotrophic cyanobacterium Anabaena sp. strain PCC 7120 had nifHDK transcription induced under an iron-depleted condition that made cells susceptible to oxidative stress from ROS (26, 30). Subsequently, we believe NiFHDk is expressed as an ROS-mitigating redox-balancing activity in Anabaena sp. PCC 7120, similar to what we observed here for Cyanothecae sp. 51142. Additionally, studies in purple nonsulfur bacteria have shown that when CO₂ fixation is blocked, dinitrogenase is expressed under N-replete conditions to allow for the oxidation of NADH so metabolism can proceed under balanced redox conditions (31, 32).

Photosynthetic electron transfer during nutrient limitation. The IM-RP labeling of proteins involved in linear, cyclic, and alternative electron flow (LEF, CEF, and AEF, respectively), respiration, and antioxidant strategies displays diverse trends (Fig. 4). It is vital that cells can initiate fast and flexible adjustments to electron flow during nutrient limitation (7, 10). Labelling of proteins involved for each of these processes has distinct implications on the redox state of Cyanothecae sp. 51142, as LEF, CEF, and respiration processes result in net-positive, net-zero, and net-negative formation of reducing equivalent, respectively. LEF transports electrons for CO₂ fixation, while CEF around PSI is thought to balance the ATP/NADPH budget of photosynthesis (10), and respiratory enzymes obtain electrons from carbohydrate catabolism and deliver electrons to plastoquinone (PQ) (Fig. 5) (33, 34). AEF proteins include the family A flavoproteins (Flv1 to Flv4), which remove electrons from LEF to prevent cell overreduction (6, 35), and thioredoxins, ubiquitous enzymes with a conserved disulfide motif, which reduce proteins for myriad purposes, including activation, regulation, and reducing antioxidants, such as peroxiredoxin (28, 36). Last, hydrogenases, including Hox, Hup, and NiFHDk, can allow for H₂ production to proceed to H₂ (1, 6). Hox and Hup are not identified in either IM-RP or global protein data, consistent with a previous study of aerobic H₂ production in Cyanothecae sp. 51142 (37). If NiFHDk-mediated H₂ production operates as a sink for excess electrons from the cell to protect it from redox imbalance, the activity of bidirectional hydrogenase-driven electron scavenging would be counterproductive.

Little is known about the signaling mechanism behind the induction of electron flow strategies, although recently, Strand et al. found that elevated H₂O₂ activated CEF in plants either directly through redox modulation of enzymes, or indirectly by affecting other photosynthetic processes (10). NADP(H) dehydrogenase 1 (NDH1) was implicated as a potential target because NDH1-deficient mutants did not have elevated levels of CEF following in vivo H₂O₂ treatments, and because NDH transcript levels increased post-H₂O₂ treatments in wild-type study organisms (10, 38). Protein redox and ROS signaling are both viable candidates for quick and reversible activation of any of the AEFs during environmental perturbation and nutrient flux. Redox probe chemoproteomic results showed NiFHDk, thioredoxins (TrxA and TrxB), peroxiredoxin (cce_3126), and subunits of NADP(H) dehydrogenase (NDH1) as having highly reduced patterns in both time course studies, while ferredoxin-NAD(P)H-reductase (FNR/PetH) and cytochrome b₅f subunit (PetB) were highly reduced under the CO₂-depleted condition (Fig. 4). These trends may be
due to two potential physiological features: (i) heavily reduced proteins might signify the cells’ reductant allocation strategy, or (ii) heavily reduced proteins might point toward enzymes that create or are affected by electron build-up due to bottlenecks within electron flows. These bottlenecks may occur at various degrees along the electron transport chain, including the slow diffusion of PQ, translocation to PetB, the turnover of ATP synthase, or from dissymmetry of the 3:1 ATP and NADPH stoichiometric requirements for the Calvin-Benson cycle (7).

**Cellular tactics and strategies for dealing with ROS.** ROS form during photosynthesis when the intensity of light-driven electron transport outpaces the rate of electron consumption. This is especially problematic during nutrient limitation, which creates redox imbalance due to the overreduction of electron transport proteins (9, 22, 26). Under the specified culture conditions, the flavoprotein Flv1-Flv3 heterodimer (Flv1/3) appears to be another vital enzyme working to balance redox in *Cyanothece* sp. 51142. This complex reduces PSII-evolved O2 directly to H2O in a modified Mehler reaction (35, 39, 40) and may work to achieve a less oxic environment, allowing for NifHDK to function in aerobic environments. Allahverdiyeva et al. found that Flv1/3 used ~20% of the PSII-generated electrons to reduce O2 to H2O under normal conditions, but these rates increased to 60% under extreme CO2 limitation (41). Flv1 and Flv3 were identified in every time point under both conditions in the global protein data (see Data Set S3 in the supplemental material), while Flv3 was labeled by IM-RP, and a site of labeling was identified (residue C410) (see Data Set S1 in the supplemental material); these observations allude to the possibility of regulation of Flv1/3 through redox events sensed by Flv3. Ermakova et al. demonstrated that Flv3b, a heterocyst-specific flavodoxin, enables nitrogenase activity in oxic environments in heterocysts (42); this finding supports our own involving Flv3.

Additionally, superoxide dismutase (SOD) was highly abundant in the global protein abundance data (see Data Set S3 in the supplemental material), as was the peroxiredoxin cce_3126, which plays a significant role in *Cyanothece* sp. 51142 ROS alleviation under diazotrophic conditions (37). cce_3126 remained highly reduced, as confirmed by probe labeling, signifying that it was actively poised in a reduced state. Two sites of labeling were also identified (residues C141 and C156) for this peroxiredoxin.

**Intracellular communication and the role of redox reactions.** It is proposed that redox-dependent signaling is the core of photosynthetic regulation, and that other control mechanisms either function in conjunction with redox control or act as ancillary measures modulating redox signaling pathways (43). A substantial group of enzymes responsible for sensing and regulating metabolic activity or transcription were labeled by the probes (see...
Table S1 in the supplemental material), indicating that redox sensitivity may be a molecular feature for sensing the environment or receiving signals prior to performing regulatory functions.

As described earlier, we labeled multiple thioredoxins with IM-RP; these enzymes play diverse roles in regulating protein function. We evaluated the protein-protein interactions for the m-type thioredoxin, TrxA, using STRING (44), and the results were cross-analyzed with the IM-RP-labeled protein data. Each of the TrxA interaction partners was labeled by IM-RP (the dynamic redox profiles for these interaction partners can be found in Fig. S2 in the supplemental material). Proteins involved in more specific regulatory or signaling roles and identified by IM-RP include the regulator RpaB, which has been shown to decrease the efficiency of energy transfer from the phycobilisomes to PSII relative to PSI in Synechocystis sp. PCC 6803 (45). The transcription regulator cyA-brB (cce_0453) was only probe labeled in the C-depleted experiments. This protein has been implicated in multiple roles, including in modulation of the expression of genes involved in low CO₂ uptake (46) and the repression of Hox activity in Synechocystis sp. PCC 6803 (47, 48). The regulator CalA (cce_3240) was labeled in both experiments and has been shown to repress the transcription of hypC, which is required for synthesis of a functional hydrogenase (49). As previously mentioned, the hydrogenases Hox and Hup were not identified in the global or probe-labeled protein data.

**Conclusion.** Herein, we present experimental evidence demonstrating an extension for the biological function of the dinitrogenase NifHDK as an enzyme capable of providing protection from oxidative stress by helping to balance the redox state of *Cyanothece* sp. 51142. In order to study cellular redox profiles during H₂ production, *Cyanothece* sp. 51142 was grown under N-starved photosynthetic conditions. H₂ production was sustained for over 40 h, and the ROS levels and protein redox profiles were captured throughout the time course by chemoproteomics and correlative measurements. In general, the redox imbalance of the cell caused by photosynthetic water splitting exceeding electron carrier capacity, a condition exasperated by nutrient limitation, extended broadly to proteins sensitive to redox and also increased ROS levels as O₂ was reduced to harmful intermediates. In response to this situation, our results indicate that NifHDK produced H₂ to oxidize the overly reduced intracellular environment, thereby permitting continued metabolic reactions and protection from ROS damage. Furthermore, our data provide evidence that multiple transcription and activity regulators are redox sensitive, and future analysis could illuminate specific signaling strategies in *Cyanothece* sp. 51142 that may be key to cell metabolism. *Cyanothece*

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**FIG 5** Electron transport and partitioning in *Cyanothece* sp. 51142. Enzymes in red were labeled by IM-RP; enzymes in black were only found in the global proteomics data. Electrons flow from photosystem II (PSII) to photosystem I (PSI) through plastoquinone (PQ) to cytochrome b/f complex (Cyt b/f), cytochrome c₆ (Cyt c₆), or plastocyanin (PC), and then PSI. From PSI, electrons are transferred to ferredoxin (Fd), ferredoxin:NADP⁺ reductase (FNR), and then NADP⁺. NADPH is used for CO₂ fixation (when CO₂ is available) and other metabolic processes. In cyclic photosynthetic electron transport, electrons can flow from Fd to PQ, or NDH to PQ. Proteins involved in respiratory electron transfer to PQ are represented as orange shapes. NADH dehydrogenases (NDH) and succinate dehydrogenase (SDH) can supply the PQ pool with electrons derived from carbohydrate catabolism. Cytochrome oxidase (COX) can be reduced by Cytc6/PC and reduce O₂ to H₂O₂. Proteins involved in alternative electron acceptors are represented as purple shapes. Flavodoxin (Flv) 1/3 performs a modified Mehler reaction by reducing O₂ directly to H₂O. Thio...
sp. 51142 uses an array of strategies to maintain redox homeostasis during nutrient limitation; diverting and manipulating photosynthetic electron flow could prove to be a potential strategy for increasing photosynthetic H₂ production for biofuel technology development. Finally, our probe data reveal redox dynamics and Cys sites of probe labeling indicating a likely modulation of NiFHDK activity by redox, a feature that may become useful for engineering efficient H₂ production with improved output.

**FUNDING INFORMATION**

This work, including the efforts of Natalie Sadler, Hans Bernstein, Matthew R. Melnicki, Moiz Charania, Eric Hill, Lindsey Anderson, Matthew E. Monroe, Richard Smith, Alexander S. Beliaev, and Aaron T. Wright, was funded by U.S. Department of Energy (DOE).

This research was supported by the Genomic Science Program of the U.S. DOE-DOE and is a contribution of the PNNL Biofuels and Foundational Scientific Focus Areas. MS-based proteomic measurements used capabilities developed partially under the GSP Panomics project; MS-based measurements and microscopy were performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by OBER at PNNL.

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Hydrogen Production Alleviates Oxidative Stress


