Overexpression, Isolation, and Spectroscopic Characterization of the Bidirectional [NiFe] Hydrogenase from Synechocystis sp. PCC 6803

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The bidirectional [NiFe] hydrogenase of the cyanobacterium Synechocystis sp. PCC 6803 was purified to apparent homogeneity by a single affinity chromatography step using a Synechocystis mutant with a Strep-tag II fused to the C terminus of HoxF. To increase the yield of purified enzyme and to test its overexpression capacity in Synechocystis the psbAII promoter was inserted upstream of the hoxE gene. In addition, the accessory genes (lypF, C, D, E, A, and B) from Nostoc sp. PCC 7120 were expressed under control of the psbAII promoter. The respective strains show higher hydrogenase activities compared with the wild type. For the first time a Fourier transform infrared (FTIR) spectroscopic characterization of a [NiFe] hydrogenase from an oxygen phototroph is presented, revealing that two cyanides and one carbon monoxide coordinate the iron of the active site. At least four different redox states of the active site were detected during the reversible activation/inactivation. Although these states appear similar to those observed in standard [NiFe] hydrogenases, no paramagnetic nickel state could be detected in the fully oxidized and reduced forms. Electron paramagnetic resonance spectroscopy confirms the presence of several iron-sulfur clusters after reductive activation. One [4Fe4S] \(^\text{2+}\) and at least one [2Fe2S] \(^\text{2+}\) cluster could be identified. Catalytic amounts of NADH or NADPH are sufficient to activate the reaction of this enzyme with hydrogen.

Hydrogenases are metalloenzymes that catalyze the reversible cleavage of \(\text{H}_2\) into two protons and two electrons. Three types of hydrogenases are recognized, two contain a binuclear metal center ([FeFe] or [NiFe]) and the third type harbors a mononuclear iron center. Despite being unrelated in an evolutionary context (1, 2) all three classes share a Fe(CO) \(_n\) nuclear iron center. Despite being unrelated in evolutionary context (1, 2) all three classes share a Fe(CO) \(_n\) nuclear iron center. The active site in [NiFe] hydrogenases has been characterized as a NiFe(CN) \(_2\)(CO) center by x-ray crystallography (22, 23) and by Fourier transform infrared spectroscopic
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(FITIR)$^2$ studies (4, 21, 31–33), showing that the iron atom carries three inorganic diatomic ligands, two cyanides and one carbon monoxide. Nickel is coordinated by the sulfur atoms of four cysteines. Two of them are linked to the iron. In aerobically isolated enzymes, crystallographic studies indicate a third bridging ligand, which is a mono-oxo (hydroperoxide) ligand in the Niu-B (ready state), whereas a bridging di-oxo (hydroperoxide) species has been suggested in the Niu-A (unready state) (34, 35). The exact nature of the ligand di-oxo (hydroperoxide) species has been suggested in the Niu-A state requires hours (4, 19–21). Both in vivo and in vitro studies have shown that the enzyme is inactive but can be activated by reduction with hydrogen. Upon activation the oxygen ligand is removed (36, 37). The two states Ni$_{\alpha}$-B and Ni$_{\alpha}$-A differ in their activation kinetics. Ni$_{\alpha}$-B activation takes place within a time frame spanning from seconds to minutes, whereas the Ni$_{\alpha}$-A state requires hours (4, 19–21). Both states can be monitored with electron paramagnetic resonance (EPR) spectroscopy, via the low spin Ni$^{3+}$ ion. In one-electron reactions these states are converted to their respective reduced states, Ni$_{\alpha}$-S and Ni$_{\alpha}$-S, which are also catalytically inactive, but EPR-silent. All redox states can be distinguished by FTIR spectroscopy via their characteristic band positions of the CO and CN stretching vibrations (4, 21, 26, 32). For an overview about the different states of standard [NiFe] hydrogenases, see Refs. 4 and 29. It is proposed that at least three of the various redox states identified in [NiFe] hydrogenases are directly involved in the catalytic cleavage and formation of H$_2$. Ni$_{\alpha}$-S (EPR-silent) is the most oxidized that is converted by reduction to the intermediate Ni$_{\alpha}$-C (EPR-active), which is then fully reduced to Ni$_{\alpha}$-SR (EPR-silent). Each of the one-electron reduction steps is accompanied by a proton transfer step. Although nickel cycles between diamagnetic +II and paramagnetic +III states, the iron in the active site remains in its valence state during catalysis (25, 38). The splitting of H$_2$ is known to be a heterolytic process ($H_2 \rightarrow H^- + H^+$) (3) and the electrons are believed to be transferred via FeS clusters between the active site and the redox partners of the enzyme.

The bidirectional, soluble NAD$^+$-reducing hydrogenase from Re H16 (SH) is a close relative of the cyanobacterial bidirectional hydrogenase and the best characterized of its class. For this enzyme a non-standard coordination of the active [NiFe] site was proposed with one additional cyanide ligand bound to each iron and nickel (15, 16, 39). Such a ligation would possibly protect the catalytic center from binding of oxygen and the related inactivation. The protein was activated by hydrogen in the presence of catalytic amounts of NADH or NADPH (16). Under such conditions, no evidence of paramagnetic nickel species could be detected by EPR spectroscopy (16). However, in some enzyme preparations significant amounts of Ni$_{\alpha}$-C could also be induced electrochemically or by an excess of NADH or dithionite (39).

Oxygenic photosynthetic microorganisms are a matter of intense interest for the production of hydrogen by solar power. The bidirectional hydrogenase is the enzyme naturally involved in this process in cyanobacteria (10–12). In this work we present a newly developed rapid and gentle purification protocol for Synechocystis sp. PCC 6803 and the first characterization of the enzymes active site and its iron-sulfur centers by a combination of FTIR and EPR spectroscopy.

MATERIALS AND METHODS

Cell Growth—The wild type strains Synechocystis sp. PCC 6803, Nostoc sp. PCC 7120, and the Synechocystis mutants were grown in BG-11 (40) supplemented with 5 mm TES, pH 8, at 28 °C and 50 μE m$^{-2}$ s$^{-1}$ bubbled with air. For purification, Synechocystis mutant strain E3 was cultured in 5-liter glass bot-
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**TABLE 1**

Primer sequences used during cloning procedures

| Primer   | Sequence 5’–3’                  |
|----------|---------------------------------|
| Anahyp1  | CATATGCGGCACTCGAGGAATTCG       |
| Anahyp2  | TATAGGCTGCAATGTTGAAGAAAATCGGTCAC |
| E-in1    | GGTTCGCTACTATGTGAGAACTGAGACCT |
| E-out1   | TCTGACAATGTAGAACAGAC           |
| E-out2   | AACTTACTTCAACACCAAGGTGG        |
| Gm1      | GTCGACAAGAGAAGACCAACCC         |
| Gm2      | GTCGACGATAGTAGGGGACCC          |
| HoxF-in1 | GAACTGCGGGTGGCTCCAGC           |
| HoxF-out1| CTATTCTGAAGGGGAAGCTA           |
| HoxF-in2 | GACCCGCACCGTGACTGATTGGGAGAGCCT |
| HoxF-out2| CAGTGGCTGTTGAATAATTCT          |

**Enzyme Assays**—$H_2$ production was measured with a Clark-type electrode (43) in the presence of $5 \text{mM}$ methyl viologen and $10 \text{mM}$ sodium dithionite (10). Protein concentrations were determined by the Bradford assay (Bio-Rad, Laboratories) using bovine serum albumin as a standard (44).

**Immunoblot Analysis**—Proteins were separated by electrophoresis in 16.5% Tricine-SDS gels (45), and either stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes (Porablot, Macherey-Nagel, Düren, Germany). Proteins were detected with antibodies raised against *Synechocystis* HoxF (1:1000) and HoxH (1:100) (10) or against the HypD (1:500) (46) of *R. eutropha* and the ECL system (Amersham Biosciences). Strep-tag II antibodies, obtained from IBA (Göttingen, Germany) were used as described by the manufacturer. As a protein marker, the prestained protein ladder PageRuler (Fermentas, St. Leon-Rot, Germany) was used.

**MALDI-MS Analysis and Edman Degradation**—The excised gel slices were bleached, reduced with dithiothreitol, and acetylated with iodacetamide. After digestion with trypsin, all mass spectra were acquired with a MALDI-TOF mass spectrometer (ABI Voyager-STR). The measurements were carried out with an $\alpha$-cyano-4-hydroxyccinnamic acid matrix. They were calibrated externally and internally using a standard Sequazyme Peptide Mass Standard kit and by the peptides generated by the autotryptolysis of trypsin. Protein identification by mass spectrometry data was achieved using the Protein Prospector MS-Fit program (University of California, San Francisco, CA) and the Mascot search engine (version 2.0, Matrix Science Ltd.).

**Sample Treatment**—Protein samples were filled in X-Band EPR tubes (Rototemp Spintec 707-SQ-250). For reductive activation catalytic amounts (5 mol % of protein) of NADH or NADPH were added and the samples were flushed with 100% $H_2$ gas for 30 min in a glove box with an anaerobic atmosphere (5% $H_2$, 95% $N_2$). Sodium dithionite solution was prepared in an anoxic buffer, and the reduction was carried out under an argon atmosphere by adding a 20-fold excess to the sample. Furthermore, excess NADH was added to another sample that had been incubated with hydrogen and frozen after 30 min. In order to produce the highest oxidized state(s) in the enzyme, experiments with an excess of 2,6-dichloroindophenol were also carried out. Aliquots of the protein samples after various chemical treatments. The cells were harvested at 20 min by centrifugation at 6,000 × g. 

**Cloning Procedures**—DNA cloning and PCR amplification were performed using standard procedures (41). All primer sequences used during the cloning procedure are listed in Table 1. To insert a pbsAll promoter upstream of the hoxE gene from *Synechocystis*, a PCR product was amplified using the primer pairs E-out1/E-in1 and E-in2/E-out2. The gentamycin cassette that had been amplified with primers Gm1 and Gm2 was then fused to these two PCR products as described (42). The resulting product was ligated into pCRII-TOPO (Invitrogen). In the final step this vector was cut by KpnI and NdeI, and the pbsAll promoter of pDH1 (42) was excised with the same enzymes and inserted into these sites.

For construction of the Strep-tag II mutant (E3), the Strep-tag II sequence with an Alalin-Serin-linker (Fig. 2) was fused to the C terminus of the HoxF protein. Then, two PCR products from the genomic DNA of *Synechocystis* PCC 6803 were amplified using primer pairs HoxF-out1/HoxF-in1 and HoxF-in2/HoxF-out2. The overlapping parts of the Strep-tag II sequence in these products could be fused together with a second PCR. Primer HoxF-in2 contained the restriction site for the enzyme BamHI, at this site the kanamycin resistance cassette from pUC4K was inserted as a selection marker in the same orientation as hox genes. To get a hyp gene expression construct, the respective operon of *Nostoc* sp. PCC 7120 was amplified by PCR using primers Anahyp1 and Anahyp2. The resulting 6.7-kb PCR product was cloned downstream of the pbsAll promoter into *Synechocystis* expression vector pDH2. The latter vector was constructed by cutting the kanamycin cassette out of pDH1 with restriction enzymes Xhol and Sacl, and ligating the chloramphenicol resistance cassette from pKS-CAT (HindIII and Sacl digested) into the pDH1 vector in a half-blunt end ligation reaction. This construct was transformed into the Strep-tag II mutant E3. All constructs were sequenced before transformation in *Synechocystis*.

**Enzyme Purification**—All purification steps were carried out at 4 °C under aerobic conditions. After washing and resuspending the pellet in buffer W (100 mM Tris-HCl, 150 mM NaCl, pH 8.0) the cells were disrupted by three passages through a chilled French pressure cell (Sim Amicon) at 20,000 p.s.i. To obtain the soluble fraction the extract was centrifuged at 24,000 × g for 1 h. Subsequently, a concentrated ammonium sulfate solution was slowly added to a final concentration of 20%. After centrifugation for 30 min at 11,000 × g, the supernatant was applied to a 5-ml gravity flow Strep-Tactin-Sepharose column (IBA, Göttingen, Germany). Unbound proteins were removed by washing 5 times with 1 column volume of buffer W. Recombinant protein was eluted by adding buffer E (buffer W with 2.5 mM desthiobiotin (IBA, Göttingen, Germany)) and the elution fraction was concentrated by centrifugation at 7,500 × g in centrifugal filters (Amicon Ultra 4, 10 K, Millipore, Eschborn, Germany).
Infrared Spectroscopy—Infrared spectra were recorded on a Bruker Tensor 27 FTIR spectrometer equipped with a liquid nitrogen-cooled MCT detector at a spectral resolution of 2 cm\(^{-1}\). The sample (0.1–0.6 mM protein) was held in a temperature-controlled (10 °C), gas-tight liquid cell (volume ~7 μl, path length ~50 μm) with CaF\(_2\) windows, whereas the sample chamber was purged with dried air. To follow the inactivation process after various chemical reactions, spectra were collected subsequently as a function of time while allowing air to penetrate slowly into the liquid cell.

Subsequently, the FTIR spectra were baseline corrected by means of a spline function implemented within OPUS 4.2 software supplied by Bruker. The spectra shown in this work were normalized with respect to the integral intensity of the CO stretching bands.

EPR Spectroscopy—X-Band EPR measurements at 9.5 GHz were carried out on a Bruker ESP300E spectrometer equipped with a rectangular microwave cavity working in the TE\(_{102}\) mode. The samples were placed in an Oxford ESR 900 helium-flow cryostat controlled with an Oxford ITC502 to allow measurements at temperatures between room temperature and 4 K. The microwave frequency was detected with an EIP frequency counter (Microwave Inc.). To obtain accurate g values the magnetic field was calibrated with an external standard (lithium particles embedded in LiF matrix) with a known g value of 2.002293 (47). Simulations of the spectra were performed with the MATLAB toolbox EasySpin (48). Background corrections were done by subtracting a spectrum containing only buffer solution from the spectrum of hydrogenase that was measured under the same conditions. For absolute quantification, the double-integrated spectra were compared with the spectra of a CuSO\(_4\) standard of known concentration.

RESULTS

Overexpression—The genes of the bidirectional [NiFe] hydrogenase from Synechocystis PCC 6803 (hoxE, hoxF, hoxU, hoxY, and hoxH) are located in one operon (28, 30). In the gene cluster three open reading frames of unknown function (open reading frames 3, 6, and 7) are situated (Fig. 2). Six accessory proteins HypA-F (hydrogenase pleiotropic proteins) and one protease (HoxW) are necessary for post-translational processing of the hydrogenase (42, 49). To yield a sufficient amount of the [NiFe] hydrogenase for its characterization and to test the overexpression capacity in Synechocystis PCC 6803, the pbsAll-promoter was inserted upstream of the hoxE gene in Synechocystis PCC 6803, the pbsAll-promoter upstream of the hox operon and Strep-tag II fused to the C terminus of HoxF. Cells were cultivated at 50 μE m\(^{-2}\) s\(^{-1}\) (black bars) and 200 μE m\(^{-2}\) s\(^{-1}\) (white bars). Triplicate measurements are shown; error bars represent the standard deviation.

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FIGURE 4. Hydrogenase activity as measured in the presence of methyl viologen and dithionite (A) and Western blot analysis with antibody against HypD (B). WT, Synechocystis sp. PCC 6803; E3, mutant with psbAll promoter upstream of the hox operon and Strep-tag II fused to the C terminus of HoxF; B and F, two E3 mutants with the hyp operon from Nostoc sp. PCC 7120 under the control of the psbAll promoter; HypD, R. eutropha strain HF632, which harbors the maturation proteins of the membrane-bound hydrogenase operon, as the positive control. ΔHyp, strain HF632 with deletion of the complete hyp region, as the negative control. The protein of higher molecular weight detected in the cyanobacterial strains is due to an unspecified reaction of the antibody because it is not visible in the R. eutropha strains. Triplicate measurements are shown; error bars represent the standard deviation.

encodes for the D1 protein of photosystem II, which is damaged at a higher pace at high light intensities because of stronger photodestruction. Consequently, the cells increase the expression of this gene (50).

We supposed that expression of the normal wild-type level of hyp genes might limit the formation of an active hydrogenase and that their overexpression could increase its yield. The hyp genes in Synechocystis are spread over the chromosome as single genes and are localized in different gene clusters (42). Because of this constraint, we overexpressed hyp genes (hypF, C, D, E, A, and B) from Nostoc sp. PCC 7120, which are in a single gene cluster, under the control of the psbAll promoter in the Synechocystis mutant containing the Strep-tag II and the psbAll promoter upstream of the hox operon. The result is presented in Fig. 4. Insertion of the hyp operon from Nostoc (see mutants B and F) led to a higher hydrogenase activity compared with the Synechocystis wild-type and compared with the mutant without this hyp operon. The expression of Hyp proteins from Nostoc sp. PCC 7120 in Synechocystis was confirmed by Western blot using an antibody against HypD. The crude extracts of mutants B and F contained more HypD than wild-type or the E3 mutant.

Purification—The soluble [NiFe] hydrogenase of the cyanobacterium Synechocystis sp. PCC 6803 could be purified to apparent homogeneity by affinity chromatography using a Synechocystis mutan with a Strep-tag II fused to the C terminus of HoxF. In the elution fraction of the purified enzyme all five subunits (HoxEUFYH) could be detected by Tricine-SDS-PAGE (Fig. 5). The identity of these bands was confirmed by MALDI-mass spectrometry (data not presented). Edman degradation in combination with sequencing of the purified enzyme established a subunit stoichiometry of HoxEUFYH. All subunits had unblocked N termini and could be sequenced, and aside from HoxF all subunits were detected without the N-terminal methionine. The unique amino acids occurring in five cycles (5–8, 10) of the degradation could be used to establish a ratio of 0.2:2:2:1:1 for E:F:U:Y:H (supplemental Table S1).

A complete small-scale purification procedure is summarized in Table 2. Beginning with 7.5 g of cells (wet weight) 13 μg of enzyme could be gained. The specific activity of the isolated fraction was 87.78 units/mg of protein with methyl viologen as the electron donor. The enzyme was purified 1155-fold. This high purity of the preparation could be confirmed by silver staining of the elution fraction (Fig. 5). For spectroscopic investigation the purification process was scaled up.

EPR Spectroscopy—In the isolated form of Synechocystis hydrogenase small signals attributable to an oxidized [3Fe4S]+− cluster and an organic radical (probably chlorophyll impurities) were seen (supplemental Fig. S1). However, signal intensity did not correspond to stoichiometric amounts. The overall signal intensity corresponded to 0.05 spins/protein, indicating that the [3Fe4S] signal might arise from an oxidative damaged [4Fe4S] cluster in a small fraction of the sample during purification. Addition of an excess of 2,6-dichloroindophenol, to obtain the highest oxidized state of the enzyme, did not change the overall shape of the spectrum (supplemental Fig. S1) indicating that this hydrogenase does not harbor a [3Fe4S]+− cluster as found in standard [NiFe] hydrogenases such as those from D. vulgaris Miyazaki F (28) or D. gigas (22). Furthermore, no traces

FIGURE 5. Electrophoresis in 16.5% Tricine-SDS gels of the purified [NiFe] hydrogenase from Synechocystis sp. PCC 6803. Coomassie Blue staining of crude extract (a), the first flow-through fraction (b), the first wash fraction (c), and the elution fraction with the highest protein concentration (d) from affinity chromatography. In e, a silver stain of the same elution fraction is shown.
of the Ni₉-B or Ni₉-A states were found in the EPR spectra. Their g values would be in the range of 2.33 to 2.01 (e.g. in A. vinosum Ni₉-B, gₓ = 2.33, gᵧ = 2.16, gz = 2.01, and Ni₉-A, gₓ = 2.32, gᵧ = 2.24, gz = 2.01 (4, 29)).

After addition of catalytic amounts of NADPH (5 mol %) and incubation with hydrogen for 30 min at room temperature, the signal of a reduced iron-sulfur cluster could be detected at 40 K (Fig. 6a). The g values of this cluster are gₓ = 2.016, gᵧ = 1.935, and gz = 1.928 with a line width of 1.5 mT. The temperature dependence and microwave power saturation behavior indicate that this cluster is most likely a reduced [2Fe2S]⁺ cluster rather than a [4Fe4S] cluster as already discussed for the FeS clusters in complex I (51). Spin quantification yields about one spin/protein, i.e. this cluster is present in virtually 100% of the protein. However, there seem to be two forms of this [2Fe2S] cluster with different gₓ values (see Table 3). This behavior is known from other iron-sulfur proteins where two forms were also observed in varying amounts depending on solvent and freezing conditions (52). Two populations with slightly different gₓ values might arise from partial protonation of an amino acid in close vicinity to the cluster. Moreover, it is also possible that two different [2Fe2S]⁺ clusters are present, which have by coincidence the same gᵧ and gz values. We are unable to distinguish these two cases, because the signal intensity corresponds only to one spin/protein.

When the temperature was decreased to 20 K signals from a second type of iron-sulfur cluster appeared with g values, gₓ = 2.04, gᵧ = 1.95, and gz = 1.88 (Fig. 6b). The line width is 5 mT and the signal could not be saturated. The saturation behavior, temperature dependence, as well as the g-anisotropy are typical for reduced [4Fe4S]⁺ clusters rather than for [2Fe2S]⁺ clusters, as found in other organisms (18, 53–55). Absolute quantification indicates about 1.9 spins/protein for the overall EPR signal, originating from both types of FeS cluster. The double-integrated simulations of both clusters have similar intensities as well as indicating the presence of both clusters in virtually 100% of the protein.

At a temperature of 10 K the spectrum becomes more complex and the lines are broadened, which is probably due to magnetic coupling of both clusters. The spin intensity still corresponds to two spins/protein and it was not possible to disentangle the spectrum with power saturation studies (supplemental Figs. S2 and S3). From this we conclude that the [4Fe4S]⁺ cluster and the [2Fe2S]⁺ cluster(s) are in spatial proximity to each other.

Because up to eight iron-sulfur clusters are expected in this protein (supplemental Table S2) (8), different reduction methods have been performed. Surprisingly, all of them produced the same signals (see Fig. 7). Besides reductive activation with catalytic amounts of NADPH and hydrogen incubation, reduction was also performed with catalytic amounts of NADH and H₂ (Fig. 7b). Also incubation of excess NADH and hydrogen did not change the shape of the signal, only an increased amount of a flavin radical was observed centered around g = 2.003 (see inset Fig. 7). This radical is visible at higher temperatures, e.g. 230 K, and has a line width of 1.9 mT. To reduce all cofactors the sample was treated with excess sodium dithionite. However, no significant change of signal shape or intensity was observed. Therefore, it is obvious that the enzyme was already in the catalytically active state.

TABLE 2
Small-scale purification of the [NiFe] hydrogenase from Synechocystis sp. PCC 6803 (1 unit = production of 1 µmol of H₂/min⁻¹)

| Sample              | Volume | Protein | Total protein | Activity | Total activity | Specific activity | Purification factor | Activity yield |
|---------------------|--------|---------|---------------|----------|----------------|------------------|---------------------|---------------|
| Crude extract       | 5.00   | 13.60   | 68.00         | 1.034    | 5.170          | 0.076            | 1                   | 100           |
| Affinity chromatography | 0.50   | 0.025   | 0.013         | 2.195    | 1.097          | 87.782           | 1155                | 21            |

FIGURE 6. EPR spectra of reduced Synechocystis hydrogenase (solid) together with corresponding simulations (dotted). Catalytic amounts of NADPH (5 mol %) were added to the protein solution and the sample was incubated with hydrogen for 30 min. For simulation parameters, see Table 3. a, T = 40 K, signal of a reduced [2Fe2S]⁺ is detected. The cluster appears in two different forms with slightly different gₓ values. b, T = 20 K, additional [4Fe4S]⁺ appears. c, T = 10 K, clusters are magnetically coupled and give rise to a splitting of the spectral components. Experimental conditions: 9.5 GHz microwave frequency, 1 mT modulation amplitude, 12.5 kHz modulation frequency, and 1 milliwatt microwave power.
TABLE 3
g tensor principal values observed for the iron-sulfur cluster in
Synechocystis hydrogenase (obtained by simulation)

| Cluster          | Synechocystis PCC 6803 | A. variabilis | P. furiosus |
|------------------|------------------------|---------------|-------------|
|                  | [2Fe2S] | [4Fe4S] | [2Fe2S] | [4Fe4S] | [2Fe2S] | [4Fe4S] |
| \( g_x \)        | 2.016/2.002 | 2.04 | 2.021 | 2.05 | 2.03 |
| \( g_y \)        | 1.935 | 1.95 | 1.94 | 1.93 | 1.93 |
| \( g_z \)        | 1.928 | 1.88 | 1.935 | 1.88 | 1.92 |
| Line width (mT)  | 1.5 | 5.0 | 1.928 | 1.88 | 1.935 |

\( ^a \) From Ref. 56.
\( ^b \) From Ref. 57.
\( ^c \) \( g \)-strain was included with 0.01, 0.02 for \( g_x, g_y, \) and \( g_z \).
\( ^d \) \( g \)-strain was included with 0.03, 0, and 0.025 for \( g_x, g_y, \) and \( g_z \).

By EPR spectroscopy no signals attributable to paramagnetic Ni(III) or Ni(I) species were found in the reduced enzyme (Figs. 6 and 7). Ni\(_x\)-C or Ni\(_x\)-L type signals are known from reduced standard hydrogenases (e.g., in A. vinosum at \( g \) values of Ni\(_x\)-C, 2.21, 2.15, and 2.01; and Ni\(_x\)-L, 2.26, 2.12, and 2.05, see Ref. 29).

The absence of a Ni\(_x\)-A, Ni\(_x\)-B, Ni\(_x\)-C, or Ni\(_x\)-L signal indicates that either the [NiFe] site remains under oxidative and reductive conditions in a diamagnetic Ni(II)-state, or that the nickel center couples to another nearby unpaired electron to an overall EPR-silent state. This is in agreement with earlier studies performed on the soluble hydrogenases from Anabaena variabilis (56), Pyrococcus furiosus (57), Nocardia opaca 1b (58), and A. vinosum (59).

**FTIR Spectroscopy**—With this technique it is in general possible to characterize all redox states of the active site of [NiFe] hydrogenases, whether paramagnetic or EPR-silent diamagnetic. These states can be assigned by means of the specific absorption frequencies of the diatomic ligands and their respective band shifts due to changes of the electron density and/or coordination environment (4, 21, 26, 32).

Fig. 8 shows the first FTIR spectra of the [NiFe] hydrogenase from Synechocystis sp. PCC 6803. In the oxidized, as isolated form of this enzyme, only three bands can be resolved in the spectral region characteristic for the CN (2076 and 2088 cm\(^{-1}\)) and CO (1957 cm\(^{-1}\)) stretching vibrations (Fig. 8a). This suggests a coordination arrangement as it is observed in standard [NiFe] hydrogenases, with two cyanides and one CO bound to the iron of the active site (20–22, 32).

The detectable redox states after various chemical treatments were assigned by comparison with those observed in standard [NiFe] hydrogenases and the Ni\(_x\)-C state found in certain preparations of the soluble bidirectional hydrogenase of R. eutropha. Furthermore, the redox states of the hydrogenase dimer of the soluble hydrogenase from A. vinosum were incorporated, which, however, could so far only be tentatively assigned via their CO stretching frequencies. An overview about characteristic band positions of the diatomic ligands is given in Fig. 10. Differences in the exact band location, which are also listed in supplemental Table S3, can be explained by variations in the embedding protein matrix in vicinity of the active site, see below (4, 21).

The spectrum after a treatment with 10 times excess 2,6-dichloroindophenol (not shown here) was identical with that from the as isolated sample. Therefore, we assume that this spectrum characterizes the highest oxidized state of the investigated enzyme, although EPR-silent (supplemental Fig. S1).

Because the CO and CN vibration band positions match best with the respective band positions of the diatomic ligands, as found in the Ni\(_x\)-B state of standard NiFe hydrogenases, we assigned this state as the “Ni\(_x\)-B-like” ready state. This implies that in the case of the cyanobacterial bidirectional enzyme the active site or its vicinity must be modified to suppress the appearance of an EPR-signal.

This most oxidized state of the enzyme can be easily activated by hydrogen, when catalytic amounts of NADH or NADPH are present. The corresponding spectrum after such a reductive treatment is displayed in Fig. 8a. It displays a dominating species with absorption bands at 2079/2063 cm\(^{-1}\) for the cyanides and 1955 cm\(^{-1}\) for the CO, respectively. The observed band positions, and the fact that no Ni(I/III) signals were detected in the corresponding EPR spectra, are in good agreement with the assignment to an activated, fully reduced, EPR-silent Ni\(_x\)-SR species. Incubation of the enzyme with a 20-fold excess of dithionite or NADH/NADPH in combination with hydrogen leads to the same IR and EPR spectra. This result...
confirms that the spectral data presented here describe indeed the most reduced redox state of this enzyme.

During the controlled, slow penetration of air into the IR transmission cell, another Ni$_x$-C-like, reduced species with a CO stretching vibration at 1968 cm$^{-1}$ and the related CN stretching at 2093 and 2079 cm$^{-1}$ appeared readily during its transformation from the fully reduced Ni$_x$-SR state in the IR spectra (Fig. 8b). The latter band overlaps with cyanide absorption bands of other redox states, see Fig. 9. However, so far there was no EPR spectroscopic proof for a Ni$_x$-C state, because this transitional/intermediate state, with a redox potential close to that of the Ni$_x$-SR, could not be stabilized in sufficient amounts under the applied conditions for EPR spectroscopic investigations.

Within the ongoing slow reoxidation process, the relative concentration of the Ni$_x$-C-like state decreased again and another transitional redox state was observed before the enzyme was fully and reversibly reoxidized to its initial (as isolated) state. The corresponding stretching vibrations of such a Ni$_y$-S-like, EPR-silent state were located at 1947 cm$^{-1}$ (CO) and 2078/2093 cm$^{-1}$ (CN$^-$). Adequate subtraction of the pure and enriched states of the spectra displayed in Fig. 8 reveals the individual spectra of the corresponding involved redox states as shown in Fig. 9.

**DISCUSSION**

Hydrogen production by photosynthetic organisms is a matter of interest to ensure future energy supply by alternative environmentally friendly sources. Oxygenic photosynthesis produces reducing equivalents and oxygen directly from water. Despite cyanobacteria being the only prokaryotes able to perform oxygenic photosynthesis a detailed biochemical characterization of the cyanobacterial [NiFe] hydrogenases is missing. Our results show, that the bidirectional [NiFe] hydrogenase from *Synechocystis* PCC 6803 can be purified under aerobic conditions to apparent homogeneity as a functional heteropentamer. The stoichiometry of the subunits indicate the amount of HoxF and -U is 2-fold higher as compared with the small and large subunits of hydrogenase (HoxYH). This is a surprising finding and very interesting in light of the quantification of transcript levels of their respective genes that found a 3X higher amount of hoxI compared with hoxH for *Synechococcus* sp. PCC 7942, although both are encoded in the same operon (60). The low abundance of HoxE indicates that it is either partly removed during the purification procedure or that it is only present in some of the complexes in vivo. A similar variability concerning an additional subunit, HoxI, unrelated to the cyanobacterial hydrogenases, which depends on the ionic strength of the buffers used, was also found for the hexameric SH of *R. eutropha* (61).

Anaerobic reduction of the [NiFe] hydrogenase from *Synechocystis* leads to high activity within a few seconds. To compensate for low preparation yields of the wild type hydrogenase we tried to overexpress the enzyme in *Synechocystis*. The task was challenging due to the genetic complexity of hydrogenase assembly (49). The overexpression of hox genes leads to an increase in activity (Fig. 3), but it was still below the level expected from the *psbAII* promoter, which is one of the strongest in cyanobacteria. We therefore assumed that expression of the hyp genes might be limiting. Due to a scattered distribution...
of hyp genes in Synechocystis, the clustering of hyp genes in Nostoc sp. PCC 7120, and the high sequence homology of both hydrogenases (62) an expression of the hyp operon from Nostoc in Synechocystis seemed to be reasonable. Nostoc possesses one set of hyp genes that seems to be involved in the maturation of both of its [NiFe] hydrogenases. Our results confirm the possibility of overexpressing these hyp genes in Synechocystis and their heterologous use in the maturation of its bidirectional hydrogenase (Fig. 4).

EPR spectroscopy reveals the presence of functional iron-sulfur centers after reductive activation. In our studies we found signals corresponding to one [4Fe4S] and at least one [2Fe2S] cluster. Both clusters are in close proximity at temperatures below 10 K because they are magnetically coupled. Additionally, a signal appears at higher temperatures that can be attributed to a flavin (line width 1.9 mT). All observed paramagnetic centers could be reversibly activated/inactivated.

The spectral shape at low temperatures is similar to the spectra from the cyanobacterium A. variabilis (56), the hyperthermophilic archaeon P. furiosus (57), and the NAD-linked hydrogenase from N. opaca 1b (58). In the first case signals from reduced [2Fe2S] and [4Fe4S] clusters could be identified. In the P. furiosus hydrogenase one [2Fe2S] cluster was found, which is magnetically coupled to another iron-sulfur center at low temperatures. For the N. opaca hydrogenase it could be shown that the [2Fe2S] and [4Fe4S] clusters are located in the diaphorase subunit. Because of sequence similarities between this hydrogenase and the cyanobacterial bidirectional enzyme, it seems plausible that the [2Fe2S] center is bound by HoxU, whereas the [4Fe4S] center could be bound by either HoxF or HoxU, but any final conclusion needs to await further direct experimental evidence. Interestingly in all of these hydrogenases no signals of a paramagnetic nickel species were observed, which seems to be a common feature in hydrogenases of this type.

The FTIR spectroscopic investigations suggest a standard hydrogenase-like coordination of the iron in the [NiFe] site, with one CO and two CN⁻. At least four different redox states of the active site have been identified. In particular two transitional states were detected only during the controlled reoxidation of the fully reduced species with air. In this way, a reversible inactivation of the enzyme to its initial, fully oxidized state was accomplished.

The direction and the degree of the observed blue and red shifts, especially of the CO stretching vibration within the various redox transitions, is comparable with those detected in the anaerobic [NiFe] standard hydrogenase from D. vulgaris Miyazaki F between the Ni₆-B, Ni₆-S, Ni₆-C, and Ni₆-SR states (see Figs. 9 and 10 and supplemental Table S3) (21). Taking the π-electron back-bonding character of the C≡O bond into account, the shift to lower wavenumbers from a Ni₆-B(III)-like state to a Ni₆-S(II) state should be caused by an increase of electron density at the active site. The corresponding CN stretches exhibit a weaker π-electron acceptability and shift to slightly higher wavenumbers. This effect is not unusual for redox transitions in standard [NiFe] hydrogenases (see below), as well as for CN⁻/CO containing model compounds, and could be explained by a decrease of hydrogen bonding strength between neighboring amino acids and the cyanide ligands (e.g. due to a deprotonation) (21, 63).

A comparable but inverse effect, with constant, respectively, slightly lower values for CN stretching (explainable by increase of the hydrogen bonding, e.g. due to a protonation of cyanide adjacent amino acids) is observed from the Ni₆-S(II) to Ni₆-C(III)-like transition for all standard hydrogenases (see Fig. 10 and supplemental Table S3) (21). The corresponding CO band position shifts to higher wavenumbers, reflecting a decrease of the electron density at the active site. In turn, another shift to lower wavenumbers was found for transition from Ni₆-C(III)-like to Ni₆-SR(II), which is common for various standard [NiFe] hydrogenases and found for both types of the diatomic ligands, the CO and less pronounced also for the CN stretches (4, 21, 26, 32). Slight deviations (up to ± 7 cm⁻¹) in the absolute band positions could be explained by a variation of amino acids in the proximity of the bimetallic active site.

We could not find hints of any Ni(III) or Ni(I) species by means of EPR spectroscopy for the fully oxidized and reduced species. Because it seems unlikely that in the different [NiFe] hydrogenases different redox states of the nickel ion are used during catalysis these findings indicate an efficient coupling of any Ni(III) or Ni(I) ion with another nearby paramagnetic species to an overall, EPR-silent system. Any such coupling could also explain the low amount of EPR-detectable [FeS] clusters compared with the expected ones. Our experiments do not exclude the presence of a Ni₆-C state, because the corresponding transient species was observed in the IR spectra only during slow reoxidation by air and could not be stabilized in sufficient amounts for an EPR spectroscopic investigation.

Another, unlikely explanation is, that the [NiFe] site remains in a diamagnetic Ni(II) state, whereas electron density at the active site is changed by one of the coordinating ligands to the nickel during catalysis by donation or acceptance of electron

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3 M. Pandelia and W. Lubitz, personal communication.
density. This ligand could be modified by NADH/NADPH in such a way that catalytic hydrogen cleavage can take place, involving at least two transitional states, before the enzyme is fully reduced. In this respect recent studies of an [Fe] hydrogenase complex are interesting (64). It was proposed, that the methenyl-H$_2$MP$^+$ substrate can bind to the active site and accept the hydride during catalysis to form a methylene-H$_2$MP. In this process the H$_2$ molecule might bind side-on to the low spin Fe(II), which preserves its valence state during the catalytic cycle. In general it seems that the absence of a paramagnetic nickel species is a general principle in cyanobacterial bidirectional hydrogenases because the enzyme from A. variabilis neither showed nickel-specific signals in the oxidized nor reduced state (56).

A comparison with the presently available initial IR spectroscopic data of the isolated hydrogenase HoxYH modul of the (bidirectional) HoxEFUYH-type [NiFe] hydrogenase from A. vinosum reveals similarities with respect to some of the observable redox states. Thus, inter alia in an oxidized state, an active intermediate state, and a fully reduced state, all EPR-silent, were detected. These were tentatively assigned via the CO-stretching frequencies. The corresponding cyanide absorptions, however, are not well resolved and could not be unambiguously assigned (59), see Fig. 10. Depending on the particular preparation of the A. vinosum enzyme, minor amounts of Ni$_{2s}$-A and Ni$_{2s}$-B were identified by EPR spectroscopy. However, Ni$_{2s}$-C could not be observed in any of the preparations.

The presence of a Ni(II) center in the oxidized state has been suggested for other hydrogenases, e.g. in the also bidirectional, but oxygen tolerant, soluble hydrogenase from R. eutropha H16 (15, 16, 39). In this hydrogenase, however, four instead of two CN stretching vibrational bands were detected besides one CO absorbance band. These additional IR bands were attributed to two more cyanides at the bimetallic center, one coordinated to the iron, and the other to the nickel atom. The latter was supposed to protect the active site and keep it in a Ni(II) low-spin state.

Another example where the [NiFe] active site was EPR-silent in the highest oxidized state is the cyanobacterial-like uptake hydrogenase from A. ferrooxidans in its native cell environment (66). Also in this case the presence of a different ligand at the nickel of the active site was suggested, which preserves the Ni(II) state. The FTIR spectra without additional light treatment reveals a mixture of two EPR-silent forms in air: Ni$_{2s}$-A and Ni$_{2s}$-S with CO and CN absorption bands at 1951, 1949 cm$^{-1}$ and 2082/2093, 2077/2096 cm$^{-1}$, respectively. The main difference between these states is their slow and fast sensitivity with respect to light exposure. In a hydrogen atmosphere, Ni$_{2s}$-S is fully converted to the Ni$_{2s}$-S form. The purification process of the enzyme, however, leads to a modification of the active site, which shows EPR-detectable as well as silent states, similar to those observed in anaerobic standard hydrogenases (65).

None of the mentioned hydrogenases really exhibits a close similarity to both, the observed redox behavior and the characteristic IR spectroscopic band pattern of the diatomic ligands in the hydrogenase of Synechocystis sp. PCC 6803. It reveals a standard-like coordination of the active site with respect to the number of diatomic ligands (two cyanides and one carbon monoxide) and at least four standard-like redox states. However, no nickel-specific EPR signal was detected, and catalytic amounts of NADH/NADPH as in case of the soluble, oxygen-tolerant enzyme of R. eutropha (SH) were sufficient for its activation. Thus, the cyanobacterial enzyme, with its active site structure and fast activation kinetics, is situated between anaerobic [NiFe] standard hydrogenases and the SH from R. eutropha. With respect to these properties, it is very well suited to rapidly changing conditions faced by an oxygenic phototroph. It is rapidly activated under anoxic conditions, able to produce hydrogen, and on the other hand, quickly inactivated in the presence of oxygen, so as to not waste reductive power needed for carbon fixation. Further studies, including spectroelectrochemical FTIR redox titrations of the involved states are envisaged to gain a deeper insight into the redox behavior of this enzyme.

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