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A Bacterial Electron-bifurcating Hydrogenase*

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Background: Hydrogen-dependent reduction of ferredoxin, a common "low-redox potential" electron carrier in anaerobes, is a highly endergonic reaction.
Results: The [FeFe]-hydrogenase from an acetogenic bacterium strictly requires NAD^+ for ferredoxin reduction and reduces NAD^+ and ferredoxin simultaneously.
Conclusion: The [FeFe]-hydrogenase drives ferredoxin reduction at the expense of exergonic NAD^+ reduction.
Significance: The [FeFe]-hydrogenase uses electron bifurcation for energy coupling.

The Wood-Ljungdahl pathway of anaerobic CO_2 fixation with hydrogen as reductant is considered a candidate for the first life-sustaining pathway on earth because it combines carbon dioxide fixation with the synthesis of ATP via a chemiosmotic mechanism. The acetogenic bacterium *Acetobacterium woodii* uses an ancient version of the pathway that has only one site to generate the electrochemical ion potential used to drive ATP synthesis, the ferredoxin-fueled, sodium-motive Rnf complex. However, hydrogen-based ferredoxin reduction is endergonic, and how the steep energy barrier is overcome has been an enigma for a long time. We have purified a multimeric [FeFe]-hydrogenase from *A. woodii* containing four subunits (HydABCD) which is predicted to have one [H]-cluster, three [2Fe2S]-, and six [4Fe4S]-clusters consistent with the experimental determination of 32 mol of Fe and 30 mol of acid-labile sulfur. The enzyme indeed catalyzed hydrogen-based ferredoxin reduction, but required NAD^+ for this reaction. NAD^+ was also reduced but only in the presence of ferredoxin. NAD^+ and ferredoxin reduction both required flavin. Spectroscopic analyses revealed that NAD^+ and ferredoxin reduction are strictly coupled and that they are reduced in a 1:1 stoichiometry. Apparently, the multimeric hydrogenase of *A. woodii* is a soluble energy-converting hydrogenase that uses electron bifurcation to drive the endergonic ferredoxin reduction by coupling it to the exergonic NAD^+ reduction.

Carbon dioxide fixation is the essential feature for lithoautotrophic growth and is a prerequisite for microbes to inhabit the early earth. Carbon dioxide fixation requires reducing equivalents that may have come from oxidation of gases such as hydrogen or carbon monoxide. In addition, energy has to be conserved during the process to provide the power for the synthesis of macromolecules. A pathway known to meet both needs is the Wood-Ljungdahl pathway which is considered to have evolved on earth very early. It provides two essential features for a life-sustaining process from the most basic chemical building blocks: carbon dioxide fixation that provides cells with a precursor, acetyl-CoA, for biomass production and conservation of energy in the form of ATP (1–4). The pathway involves the reduction of carbon dioxide to formate, binding of formate to the cofactor tetrahydrofolate and its subsequent reduction to methyl-tetrahydrofolate (Fig. 1). An additional mol of carbon dioxide is reduced to form enzyme-trapped carbon monoxide that is then condensed with a methyl group and coenzyme A to yield acetyl-CoA. In the anabolic route, acetyl-CoA is the precursor molecule for biosynthetic reactions (5–7). The anaerobic acetogenic bacterium *Acetobacterium woodii* uses a simple, ancient version of this pathway that combines carbon dioxide fixation with the generation and utilization of a sodium ion gradient across the cytoplasmic membrane for ATP synthesis (4, 8). That pathway does not involve cytochromes or quinones. Instead, *A. woodii* employs a sodium-motive ferredoxin: NAD^+-oxidoreductase (Rnf complex) that couples the exergonic electron flow from reduced ferredoxin to NAD^+ to establish a transmembrane electrochemical Na^+ gradient that then drives the synthesis of ATP via a well characterized Na^+ F_1F_o-ATP synthase (9–15). The genome sequence showed that the Rnf complex is apparently the only ion (Na^+) motive enzyme coupled with the process of acetogenesis, which underlines the importance of reduced ferredoxin for the energy metabolism of *A. woodii* (8).

Reduced ferredoxin not only fuels the Rnf complex but is also the electron donor for the reduction of carbon dioxide to carbon monoxide (E_o' CO_2/CO = −520 mV (16)), a key reaction in the Wood-Ljungdahl pathway. However, reduction of ferredoxin with hydrogen as reductant is an endergonic process (E_o' $H_2/2H^+$ = −414 mV; the exact redox potential of *A. woodii* ferredoxin is not known, but because the essential ferredoxin-dependent reduction step from CO_2 to CO has a redox potential of −520 mV (16) we can assume a redox potential of at least one ferredoxin in the same order of magnitude). Thus, the question is how this steep energetic barrier for electron flow from hydrogen to ferredoxin is overcome.

In methanogenic archaea a reverse electron flow driven by the transmembrane ion potential and catalyzed by the Ech hydrogenase is the driving force for this endergonic reaction (17, 18). Such an enzyme is not encoded in the genome of *A. woodii*. One candidate is a ferredoxin-reducing hydrogenase. A protein fraction enriched from the cytoplasm of *A. woodii* was

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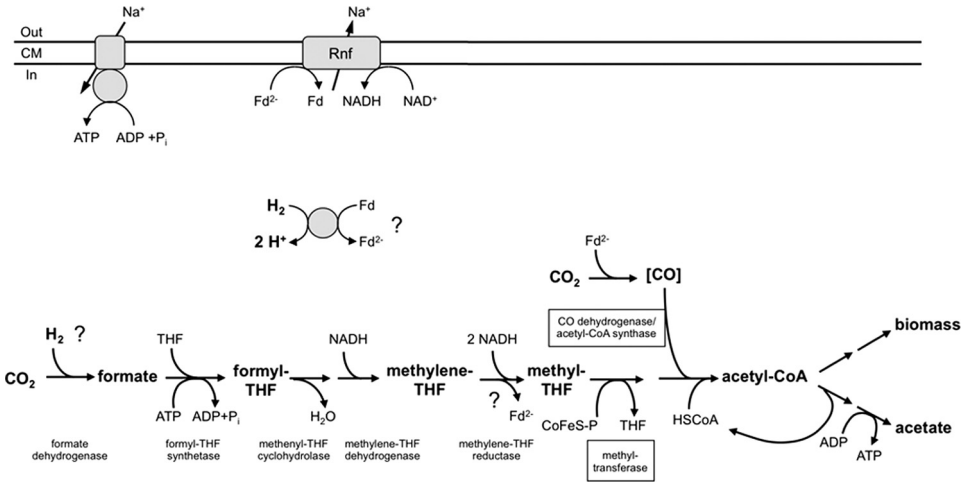

FIGURE 1. **Pathway and bioenergetics of acetogenesis as carried out by *A. woodii*.** CoA, coenzyme A; CoFeS-P, cobalt-iron-sulfur-protein; CM, cytoplasmic membrane; THF, tetrahydrofolate. *Questions marks* denote reactions in which the electron transfer has not been addressed experimentally.

indeed shown to catalyze hydrogen-dependent reduction of ferredoxin or flavodoxin, pyridine nucleotides were not tested (19). Unfortunately, the enzyme(s) involved in the reaction was (were) not identified or further characterized. The genome of *A. woodii* encodes one monomeric [FeFe]-hydrogenase that is genetically organized in the context of the formate dehydrogenase, indicating a role in formate oxidation (8). The other hydrogenase encoded is a putative multimeric [FeFe]-hydrogenase that is present in cells grown on fructose but to 3-fold up-regulated in cells grown on $H_2 + CO_2$ (8), indicating a role for the multimeric hydrogenase in hydrogen oxidation (8).

Here, we will present evidence that *A. woodii* employs a strategy different from methanogens to reduce ferredoxin with electrons derived from hydrogen. We purified the soluble, multimeric, [FeFe]-hydrogenase, and our biochemical data provide evidence that the enzyme uses the novel mechanism of electron bifurcation (20–23) to overcome the energetic barrier to reduce ferredoxin by coupling the reaction to the exergonic reduction of NAD^+.

EXPERIMENTAL PROCEDURES

Growth of Cells and Purification of the Hydrogenase—A. woodii (DSM 1030) was grown at 30 °C under anaerobic conditions in 20-liter flasks (Glasgerätebau Ochs; Bovenden-Lenglern, Germany) using 20 mM fructose to an OD_{600} of ~1.5 as described previously (24, 25). The medium and all buffers were prepared using the anaerobic techniques described previously (26, 27). All buffers used for preparation of cell extracts and purification contained 2 mM dithioerythritol and 4 μM resazurin. All purification steps were performed under strictly anaerobic conditions at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) filled with 95–98% N_2 and 2–5% H_2 as described (24). Cells of *A. woodii* were harvested and washed twice in buffer 1 (25 mM Tris-HCl, 420 mM sucrose, pH 7), resuspended in 150 ml of buffer 1, pH 8, with 500 mg of lysozyme, and incubated for 1 h at 37 °C. After centrifugation the protoplasts were resuspended in buffer 2 (25 mM Tris-HCl, 20 mM $MgSO_4$, 20% glycerol, pH 7.6) with 0.5 mM PMSF and 0.1 mg/ml DNaseI and passed three times through a French pressure cell (110 megapascals). Cell debris was removed by centrifugation at 24,000 × *g* for 40 min. Membranes were removed by centrifugation at 130,000 × *g* for 90 min. The supernatant containing the cytoplasmic fraction with ~1,300 mg of protein was applied to a Q-Sepharose high performance column (2.6 cm × 5 cm) equilibrated with buffer A (50 mM Tris-HCl, 20 mM $MgSO_4$, 50 mM NaCl, 20% glycerol, pH 7.6). Protein was eluted with a linear gradient of 150 ml from 50 mM to 1 M NaCl in buffer A. Methylviologen-dependent hydrogenase activity eluted at approximately 570 mM NaCl. Ammonium sulfate (0.8 M) was added to the pooled fractions, and these were loaded onto a phenyl-Sepharose high performance column (1.6 cm ×10 cm) equilibrated with buffer B (50 mM Tris-HCl, 20 mM $MgSO_4$, 0.8 M $(NH_4)_2SO_4$, 20% glycerol, pH 7.6). Protein was eluted with a linear gradient of 120 ml from 0.8 to 0 M $(NH_4)_2SO_4$ in buffer B. Hydrogenase activity eluted in a peak at approximately 0 M $(NH_4)_2SO_4$. Pooled fractions were concentrated using ultrafiltration in 100-kDa VIASPIN tubes (Sartorius Stedim Biotech GmbH) and applied to a Sephacryl S-300 high resolution column (1.6 cm ×60 cm) equilibrated with buffer C (10 mM Tris-HCl, 20 mM $MgCl_2$, 20% glycerol, pH 7.6) and eluted with buffer C at a flow rate of 0.8 ml/min. Hydrogenase activity eluted as a single peak. Pooled fractions were concentrated to a minimum of 10 mg/ml and stored at 4 °C.

Measurement of Hydrogenase Activity—Routine measurements of hydrogenase activity were performed at 30 °C with methylviologen as electron acceptor (10 mM) in buffer 1 (50 mM EPPS,[2] 2 mM dithioerythritol, pH 8.0) in 1.8-ml anaerobic cuvettes (Glasgerätebau Ochs) sealed by rubber stoppers, containing 1 ml of buffer 1 and H_2 at a pressure of 1.1 bar. Methylviologen reduction was monitored at 604 nm (ϵ = 13.9 mM^{-1} cm^{-1}). Physiological hydrogenase activity with NAD^+ and ferredoxin was measured in buffer 1 at 340 nm and 430 nm, respectively (ϵ_{NADH} = 6.2 mM^{-1} cm^{-1}, ϵ_{Fd} = 13.1 mM^{-1} cm^{-1}). Ferredoxin was purified from *Clostridium pasteurianum* as described (28). For inhibition studies with CO and determination of the temperature optimum, the enzyme was

[2] The abbreviations used are: EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Fd, ferredoxin.

TABLE 1
Purification of the hydrogenase

Hydrogenase activity was determined by monitoring the hydrogen-dependent reduction of methylviologen at 604 nm. The assay mixture contained 50 mM EPPS, 2 mM dithioerythritol, 10 mM methylviologen, pH 8.0, under an atmosphere of 100% hydrogen at a pressure of 1.1 bar. Measurements were performed at 30 °C in anaerobic cuvettes.

Purification step	Protein	Hydrogenase activity	Yield	Purification
	mg	*μmol H_2/min per mg*	*%*	*-fold*
Cytoplasm	1,362	113	100	1
Q-Sepharose	86.8	292	16.5	2.6
Phenyl-Sepharose	22.6	428	6.2	3.8
Sephacryl S-300	9.1	711	4.2	6.3

preincubated for 5 min with CO at 30 °C or at the temperature indicated, respectively.

Analytical Methods—Protein concentration was measured according to Bradford (29). Proteins were separated in 12% polyacrylamide gels and stained with Coomassie Brilliant Blue G250. The molecular mass of the purified hydrogenase was determined using a calibrated Sephacryl S-300 column equilibrated with buffer C (10 mM Tris-HCl, 20 mM $MgCl_2$, 20% glycerol, pH 7.6) and with Native gel electrophoresis performed under anaerobic conditions. Staining of hydrogenase activity in the Native gel was performed by incubating the polyacrylamide gel after protein separation in 50 ml of 0.1 M KH_2PO_4 containing 200 μM methylviologen and 400 μM 2,3,5-triphenyltetrazoliumchloride (TTC) in the anaerobic chamber. The atmospheric hydrogen of the anaerobic chamber serves as substrate for the reduction of methylviologen and TTC. The iron and sulfur content of the purified enzyme was determined by colorimetric methods (30, 31).

RESULTS

Purification of the [FeFe]-Hydrogenase from A. woodii—To purify the hydrogenase, we used fructose-grown cells because lithoautotrophic growth leads to insufficient cell growth and inefficient protein levels and because it was known from the proteomics data that the multimeric hydrogenase is also produced during heterotrophic growth (8). Cells were grown to the late exponential growth phase, harvested, and disrupted with a French pressure cell. Membranes were removed by ultracentrifugation, and the hydrogenase was purified from the cytoplasm by ion exchange chromatography on Q-Sepharose and phenyl-Sepharose followed by gel filtration on Sephacryl S-300. This procedure yielded an apparently homogeneous preparation. The enzyme was purified 6-fold to apparent homogeneity with an average specific activity of 760 units/mg (Table 1).

When the purified enzyme was analyzed on a nondenaturing gel, one protein of apparent mass of 300 kDa was visible. This protein had hydrogenase activity as apparent from an "in gel" activity assay (Fig. 2, *B* and *C*). When the protein was separated on a 12% SDS-polyacrylamide gel, two bands were visible after staining with Coomassie, but mass spectrometric analyses of the proteins revealed that each band contains two distinct proteins (Fig. 2*A*). The band with the apparent molecular mass of 68 kDa harbors HydA (63.6 kDa) and HydB (64.5 kDa), and the band with a mass of 13 kDa contained HydC (14.1 kDa) and HydD (14.3 kDa). Estimating the molecular mass via analytical

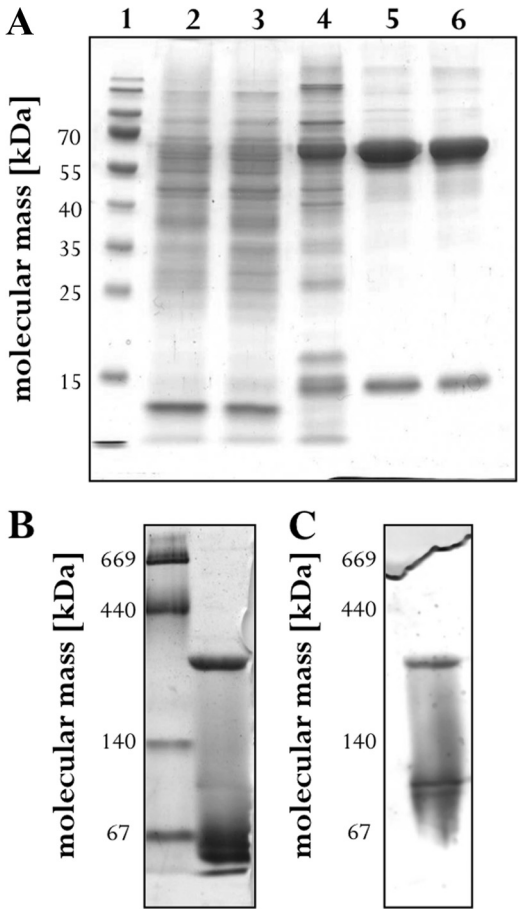

FIGURE 2. **Monitoring the purification and subunit composition of the electron-bifurcating hydrogenase.** *A*, samples of the different purification steps were separated by SDS-PAGE, and proteins were stained with Coomassie Brilliant Blue. *Lane 1*, molecular mass standards; *lane 2*, cell extract; *lane 3*, cytoplasm; *lane 4*, pooled fractions from Q-Sepharose; *lane 5*, pooled fractions from phenyl-Sepharose; *lane 6*, pooled fractions from Sephacryl S-300. 10 μg of protein was applied to each lane. *B*, purified hydrogenase was separated by Native-PAGE under anaerobic conditions and stained with Coomassie Brilliant Blue. *C*, Enzymes with hydrogenase activity were stained by incubation of the gel with triphenyltetrazolium chloride and methylviologen under an atmosphere of 3% hydrogen.

gel filtration gave a value of 260 kDa. Taken together, these data are consistent with a homodimer of a HydABCD tetramer.

Genomic Organization of the [FeFe]-Hydrogenase and Predicted Properties—With the amino acid sequence data obtained by peptide mass fingerprinting the encoding genes could be identified in the genome of *A. woodii* (8). They are present in a gene cluster that contains an additional gene, *hydE*, whose product was not detected in the purified complex. HydE has a putative histidine kinase domain and may be involved in biogenesis of the complex or gene regulation. Based on sequence analysis HydA is predicted as the catalytic subunit for hydrogen oxidation containing the H-cluster of a [FeFe]-hydrogenase, one [2Fe2S], and three [4Fe4S] clusters. HydB has a predicted flavin binding site, one [2Fe2S], and three [4Fe4S] clusters. Based on sequence similarity to the 51-kDa subunit of complex I of bacterial electron transport chains we propose that this subunit is the site of NAD^+ reduction. HydB has a C-terminal stretch with 41% similarity to ferredoxin of *Clostridium acidiurici*. *A. woodii* HydB contains 10 positively charged residues in

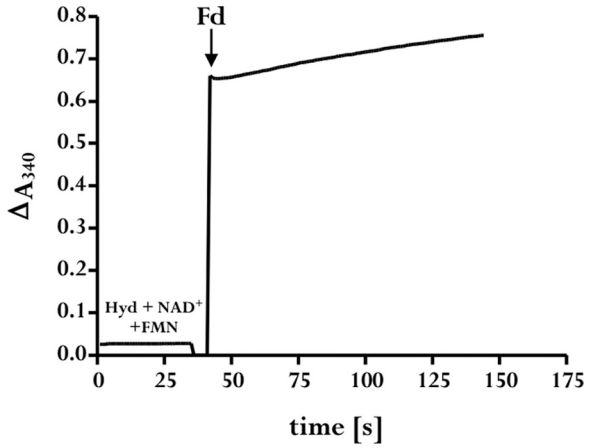

FIGURE 3. **The purified hydrogenase catalyzes reduction of NAD$^+$.** The assay mixture in anaerobic cuvettes under an atmosphere of 100% hydrogen at 1.1 bar contained 50 mM EPPS, 2 mM dithioerythritol, 50 μM FMN, pH 8.0, and 7 μg/ml hydrogenase. NAD$^+$ reduction was monitored at 340 nm. NAD$^+$ was only reduced after addition of ferredoxin (50 μM).

this 58-residue segment, whereas *C. acidiurici* ferredoxin has only one positively charged amino acid. It is possible that this site serves as binding site for the negatively charged ferredoxin. HydC is predicted to contain one [2Fe2S]-cluster, and HydD has no predicted cofactors.

Basic Biochemical Properties of the [FeFe]-Hydrogenase of A. woodii—The enzyme contained 32 ± 1.6 mol of iron/mol of protein and 30 ± 1.9 mol of acid-labile sulfide/mol of protein, which was determined colorimetrically. These data agree with the predicted three [2Fe2S], six [4Fe4S], and one H-cluster. Flavins were not detected in the purified protein. The enzyme reduced methylviologen with hydrogen as reductant with a rate of ~760 units/mg. This assay was used routinely to determine the basic biochemical properties of the enzyme.

At the optimal growth temperature (30 °C), hydrogenase activity was only 25% of the maximal activity observed at 45 °C; above this temperature, the activity declined sharply. The enzyme was inhibited by CO with 50% inhibition at ~ 0.1 nM CO in the aqueous phase, which corresponds to ~0.01% CO in the gas phase.

A Strict Coupling of NAD$^+$ and Ferredoxin Reduction—We first determined whether the enzyme can catalyze the energetic "downhill" transport of electrons from molecular hydrogen (E_o' $H_2/2H^+ = -414$ mV) to NAD$^+$ (E_o' NADH + H$^+$/NAD = −320 mV). In the absence of other electron carriers, NAD$^+$ was not reduced. Addition of ferredoxin or FMN to the assay yielded only a little NAD$^+$-reducing activity, but addition of both ferredoxin and FMN established higher NAD$^+$ reduction activity (Fig. 3), indicating that both ferredoxin and flavin are required for electron transfer from molecular hydrogen to NAD$^+$.

Enzyme kinetics were measured to determine a quantitative relationship between ferredoxin and FMN concentrations and the NAD$^+$ reduction activity. The dependence of the reaction on ferredoxin was hyperbolic with saturation at 40 μM ferredoxin, the K_m was 12 ± 3 μM (Fig. 4*A*). The dependence of the reaction on FMN was hyperbolic as well, saturation was at ~50 mM, and the K_m was 6.3 ± 1.4 μM (Fig. 4*B*).

The experiments described above clearly demonstrated NAD$^+$ reduction that was dependent on the presence of both

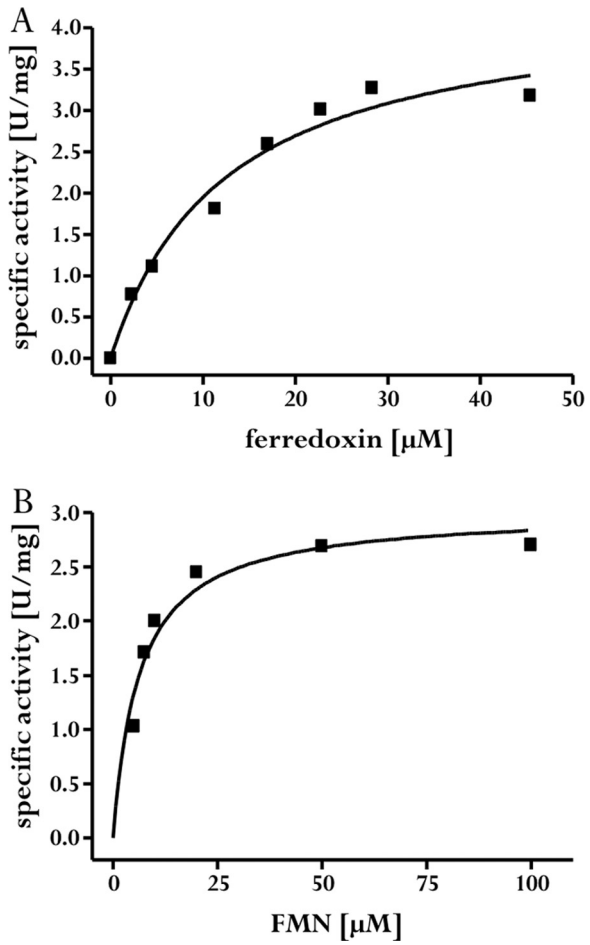

FIGURE 4. **Ferredoxin and FMN dependence of NAD$^+$ reduction activity.** Enzyme activity was measured at 30 °C in an assay under an atmosphere of 100% hydrogen at 1.1 bar in an assay mixture containing 50 mM EPPS, 2 mM dithioerythritol, 2 mM NAD$^+$, pH 8.0, and 14 μg/ml hydrogenase. For ferredoxin dependence (*A*) 50 μM FMN was added to the assay. For FMN dependence (*B*) 50 μM ferredoxin was added.

ferredoxin and FMN. Next, we asked whether the enzyme could also reduce ferredoxin. When incubated in the absence of other electron carriers, ferredoxin was not reduced. However, upon addition of both FMN and NAD$^+$, ferredoxin was reduced. The dependence of the reaction on NAD$^+$ was hyperbolic with saturation at 0.5 mM NAD$^+$; the K_m was 49 ± 18 μM (Fig. 5). The dependence of the reaction on the hydrogen concentration was also hyperbolic, the K_m was 6.3 ± 1.9% H_2 in the gas phase.

The data demonstrate that ferredoxin and NAD$^+$ were reduced by the [FeFe]-hydrogenase of *A. woodii*, and reduction of one was strictly dependent on the presence of the other electron acceptor. To address whether ferredoxin and NAD$^+$ were reduced at the same time, the isolated enzyme was incubated with NAD$^+$ and ferredoxin, and the reduction of both (with hydrogen as reductant) was monitored photometrically by measuring the absorption at 340 nm (NADH + H$^+$) and 430 nm (Fd^{2-}). Ferredoxin was indeed reduced alongside NAD$^+$ (Fig. 6). The activity was also dependent on the presence of FMN in the reaction mixture. To verify that ferredoxin was indeed reduced alongside NAD$^+$, the absorption spectrum of ferredoxin was assayed under different conditions. When ferredoxin was incubated under an atmosphere of hydrogen in the

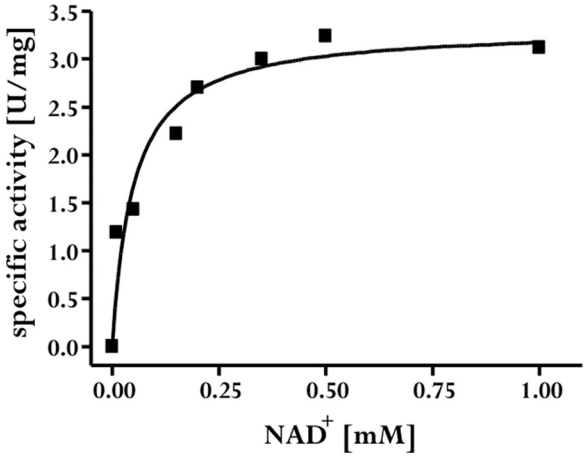

FIGURE 5. **NAD^+ dependence of ferredoxin reduction.** Enzyme activity was measured at 30 °C in an assay mixture under an atmosphere of 100% hydrogen at 1.1 bar containing 50 mM EPPS, 2 mM dithioerythritol, 50 μM ferredoxin, 50 μM FMN, pH 8.0, and 0.2 μg/ml hydrogenase. The reaction was started by the addition of NAD^+.

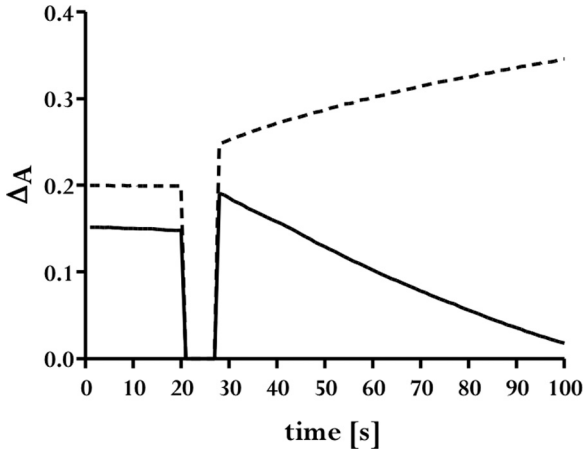

FIGURE 6. **The purified hydrogenase catalyzes reduction of ferredoxin and NAD^+ simultaneously.** Enzyme activity was measured at 30 °C in an assay mixture under an atmosphere of 100% hydrogen at 1.1 bar containing 50 mM EPPS, 2 mM dithioerythritol, 50 μM ferredoxin, 50 μM FMN, pH 8.0, and 0.2 μg/ml hydrogenase. The reaction was started by the addition of 2 mM NAD^+. Reduction of NAD^+ (*dashed line*, monitored at 340 nm) and reduction of ferredoxin (*continuous line*, monitored at 430 nm) were monitored simultaneously.

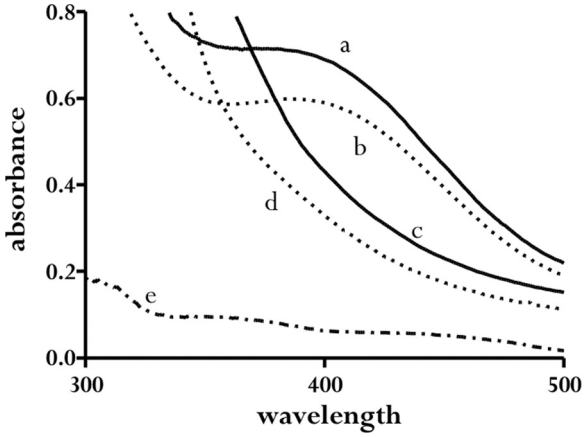

FIGURE 7. **Spectral analyses of NAD^+-dependent ferredoxin reduction.** The assay mixture contained under an atmosphere of 100% hydrogen at 1.1 bar 50 mM EPPS, 2 mM dithioerythritol, pH 8.0, and 50 μM ferredoxin, 50 μM FMN, 2 mM NAD^+, and 15 μg/ml hydrogenase. Samples were incubated for 5 min at 30 °C before spectra were monitored. Spectrum of ferredoxin, FMN, and hydrogenase (*a*); of oxidized ferredoxin (*b*); of ferredoxin, FMN, hydrogenase and NAD^+ (*c*); of sodium dithionite (0.5 mM)-reduced ferredoxin (*d*); and of hydrogenase and FMN (*e*).

presence of the purified hydrogenase, a typical absorption spectrum for oxidized ferredoxin was observed (Fig. 7). Addition of FMN did not change the ferredoxin spectrum, but additional NAD^+ led to an absorption spectrum similar to sodium dithionite-reduced ferredoxin. This experiment clearly shows reduction of ferredoxin by the multimeric hydrogenase of *A. woodii* and confirms the strict coupling of NAD^+ and ferredoxin reduction.

To determine the coupling ratio, the reduction of NAD^+ and Fd was monitored simultaneously. From these data the amount of mol NAD^+ reduced per mol of ferredoxin was calculated. From a number of experiments a stoichiometry of NAD^+:ferredoxin of 1:1 was obtained (Fig. 8).

DISCUSSION

Electron bifurcation was first demonstrated for the Etf/butyryl-CoA dehydrogenase complex of *Clostridium kluyveri* (20) in which the energetic downhill flux two-times one electron from NADH + H^+ to crotonyl-CoA drives the endergonic uphill transport of two-times one electron from NADH + H^+ to ferredoxin. The reduced ferredoxin is then used to produce hydrogen with a ferredoxin-dependent hydrogenase or to fuel the Rnf complex (21). Electron bifurcation in strictly anaerobic bacteria and archaea, as it is understood today, requires flavins. The biochemistry of the reaction is still in its infancy, and two mechanisms are discussed. One is based on the assumption that flavoproteins can exhibit three different redox potentials for the FP/FPH_2, FP/FPH, and FPH/FPH_2 with a stable semiquinone state (20). A different mechanism was proposed by Nitschke and Russel (32). In this case the flavin does not exhibit a stable semiquinone state but, instead, while in its redox-crossed-over regime the fully reduced flavin delivers its first electron in an exergonic reaction to the "low potential electron" acceptor, leaving behind a highly reactive low potential flavin that will subsequently reduce the ferredoxin in close proximity. The low potential electron acceptor thus is needed to "activate" the flavin to a high potential redox state to enable electron transfer to ferredoxin. In this reaction sequence the nominal midpoint potential for the two-electron-reduction of the left behind fully oxidized flavin is between $NADH/NAD^+$/ ($E_o' = -320$ mV) and Fd/Fd^{2-} ($E_o' \sim -500$ mV) couple thus facilitating the reduction with hydrogen ($E_o' = -414$ mV). The molecular basis for this reaction sequence has to await structural studies as much as the redox properties of the enzyme and the bound flavin need to be investigated to prove this model.

In addition to the Etf/butyryl-CoA dehydrogenase complex flavin-dependent electron bifurcation was also found for the heterodisulfide reductase in noncytochrome-containing methanogens that couple exergonic heterodisulfide reduction to the endergonic reduction of ferredoxin (22). Moreover, an electron-bifurcating transhydrogenase was described in *C. kluyveri* (23). A hydrogen-evolving hydrogenase has been described in *Thermotoga maritima* that has three subunits with similarity to the subunits of the [FeFe]-hydrogenase of *A. woodii* (33, 34). That enzyme oxidizes NADH + H^+ and produces H_2, but there was twice as much H_2 produced than NADH + H^+ consumed.

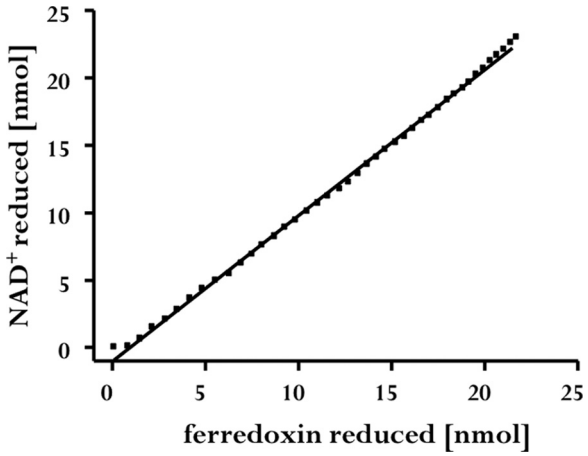

FIGURE 8. **Stoichiometry of ferredoxin and NAD^+ reduction.** The reductions of NAD^+ and ferredoxin were monitored simultaneously. 50 μM ferredoxin, 50 μM FMN, and 7 μg/ml hydrogenase were incubated until absorbance reached a constant level. The reaction was started with the addition of 2 mM NAD^+. The reduction of NAD^+ was measured at 340 nm, the reduction of ferredoxin at 430 nm. The amount of reduced electron carrier was calculated from the absorbance difference and the molar extinction coefficient. The amount of reduced NAD^+ is plotted against the amount of reduced ferredoxin.

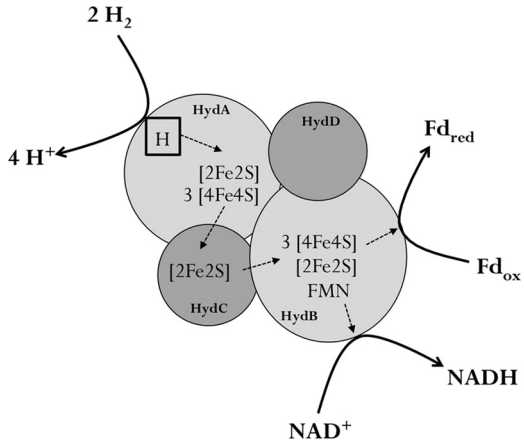

FIGURE 9. **Proposed model of the electron-bifurcating hydrogenase.** Composition of cofactors is based on sequence analysis.

Because hydrogen production from NADH + H^+ was dependent on ferredoxin, it was postulated that the "missing electrons" for hydrogen production derived from reduced ferredoxin, and a stoichiometry of Fd^{2-} and NADH + H^+ of 1:1 was proposed. It was suggested that the enzyme represents a new class of electron-bifurcating [FeFe]-hydrogenases in which the exergonic oxidation of ferredoxin ($E_o' = -453$ mV) is used to drive the unfavorable oxidation of NADH to produce H_2. Because the enzyme is a hydrogen-evolving hydrogenase that combines electrons derived from reduced ferredoxin and NADH + H^+ to reduce protons to hydrogen gas, the term electron-confurcating hydrogenase was suggested for this hydrogen-evolving hydrogenase (35).

Here, we describe an electron-bifurcating hydrogenase that apparently couples the reverse reaction, exergonic downhill flux of two electrons to NAD^+ that then drives the endergonic uphill transport of two electrons from hydrogen to ferredoxin. Two independent sets of experiments clearly show the reduction of ferredoxin and firmly establish a Fd^{2-}:NADH + H^+ stoichiometry of 1:1. The total amount of ferredoxin reduced is another argument in favor of a strict coupling. The monomeric iron-only hydrogenase of *C. pasteurianum* can also reduce ferredoxin, but only 55% of the ferredoxin was reduced under the same reaction conditions (22). A further reduction was not possible because the thermodynamic equilibrium of ferredoxin reduction with hydrogen was reached. In contrast, the hydrogenase of *A. woodii* was able to reduce almost 100% of the ferredoxin. This is far above the thermodynamic equilibrium of the uncoupled reaction at the level of sodium dithionite-reduced ferredoxin ($E_o' = -660$ mV (36)).

In contrast to the [FeFe]-hydrogenase from *T. maritima* the gene cluster of *A. woodii* has two additional genes *hydD* and *hydE* whose role is unknown. HydE is apparently not part of the purified enzyme. HydD is not predicted to have cofactors and, therefore, is unlikely involved in electron flow. HydD may have a role in assembly or stability of the complex. Based on the predicted cofactor content of the enzyme we propose that HydA is the entry point of electrons derived from molecular hydrogen. Electrons are then transferred to HydB which contains a flavin binding site and, thus, is the site of bifurcating electrons to ferredoxin and NAD^+ (Fig. 9).

A hydrogenase with genetic organization similar to that in *A. woodii* has been characterized in *Thermoanaerobacter tengcongensis*. This multimeric hydrogenase was reported to be only NAD^+-dependent, and dependence of ferredoxin or even electron bifurcation was not reported (37). Tetrameric (and trimeric) hydrogenases with the same or similar genetic organization as present in *A. woodii* are widespread in bacteria (34, 38), indicating that the electron-bifurcating hydrogenase of *A. woodii* is just the tip of an iceberg and that the same mechanism of hydrogen activation may apply to hydrogenases from many different species.

The [FeFe]-hydrogenase of *A. woodii* fills an important gap in our understanding of the bioenergetics of acetogenesis. The hydrogenase not only produces NADH + H^+, which is used as a reductant in the Wood-Ljungdahl pathway, but also reduced ferredoxin. The reduced ferredoxin then serves two functions: it is used as "fuel" for the Rnf complex and thus ATP synthesis via a sodium ion gradient and it is used as reductant for the CO_2/CO couple. Thus, a soluble enzyme overcomes the energy barrier in the first step of the electron pathway as well as one step in the carbon pathway. Because the metabolism of *A. woodii* is near the thermodynamic equilibrium with only one coupling site for the generation of an chemiosmotic ion gradient it seems reasonable not to use this gradient to facilitate the first reaction of lithoautotrophic growth. Instead, electron bifurcation drives the endergonic reaction without the need to use the energetically valuable ion gradient.

The bifurcating mechanism of the hydrogenase underlines the simplicity of the metabolism of *A. woodii*. Close to the thermodynamic equilibrium the metabolism enables carbon fixation while conserving energy without the use of cytochromes, quinones, and other membrane-soluble electron carriers. With the Rnf complex as sole membrane-bound ion pump this is the most simple way of chemiosmotic energy conservation in the Wood-Ljungdahl pathway while taking advantage of bifurca-

tion as a soluble alternative to energy coupling. The *A. woodii* version of the Wood-Ljungdahl pathway gives an answer to the question how life on earth might have evolved. The electron-bifurcating hydrogenase solves a puzzling feature in our understanding of ancient carbon dioxide fixation with hydrogen as sole source of reducing power. Further studies need to be done to shed light on the underlying molecular mechanism of electron transfer in this kind of soluble energy-coupling redox enzymes.

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