Functional Studies of [FeFe] Hydrogenase Maturation in an *Escherichia coli* Biosynthetic System

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Received 23 August 2005/Accepted 27 December 2005

Maturation of [FeFe] hydrogenases requires the biosynthesis and insertion of the catalytic iron-sulfur cluster, the H cluster. Two radical S-adenosylmethionine (SAM) proteins proposed to function in H cluster biosynthesis, HydEF and HydG, were recently identified in the *hydEF* mutant of the green alga *Chlamydomonas reinhardtii* (M. C. Posewitz, P. W. King, S. L. Smolinski, L. Zhang, M. Seibert, and M. L. Ghirardi, J. Biol. Chem. 279:25711–25720, 2004). Previous efforts to study [FeFe] hydrogenase maturation in *Escherichia coli* by coexpression of *C. reinhardtii* HydEF and HydG and the HydA1 [FeFe] hydrogenase were hindered by instability of the *hydEF* and *hydG* expression clones. A more stable [FeFe] hydrogenase expression system has been achieved in *E. coli* by cloning and coexpression of *hydE*, *hydF*, and *hydG* from the bacterium *Clostridium acetobutylicum*. Coexpression of the *C. acetobutylicum* maturation proteins with various algal and bacterial [FeFe] hydrogenases in *E. coli* resulted in purified enzymes with specific activities that were similar to those of the enzymes purified from native sources. In the case of structurally complex [FeFe] hydrogenases, maturation of the catalytic sites could occur in the absence of an accessory iron-sulfur cluster domain. Initial investigations of the structure and function of the maturation proteins HydE, HydF, and HydG showed that the highly conserved radical-SAM domains of both HydE and HydG and the GTPase domain of HydF were essential for achieving biosynthesis of active [FeFe] hydrogenases. Together, these results demonstrate that the catalytic domain and a functionally complete set of Hyd maturation proteins are fundamental to achieving biosynthesis of catalytic [FeFe] hydrogenases.

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H$_2$ metabolism in microorganisms requires the catalytic activity of hydrogenases, a specialized group of metalloproteins (29, 38, 51). The [NiFe] hydrogenases and [FeFe] hydrogenases catalyze the reaction $2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2$. A third type, the iron-sulfur cluster-free or H$_2$-forming methylenetetrahydromethanopterin dehydrogenase, catalyzes hydride transfer from H$_2$ to methenyl-tetrahydromethanopterin (29). The three distinct forms of hydrogenase have each been shown to possess a unique type of catalytic site (29, 38, 51). Maturation of the [NiFe] hydrogenases and [FeFe] hydrogenases requires a specialized set of proteins that together biosynthesize the unique catalytic metallocluster found in each enzyme family. Many details of the biochemical events of [NiFe] hydrogenase maturation have been elucidated through characterization of the maturation proteins that function in biosynthesis of the catalytic [NiFe] cluster (8, 10, 12, 28, 37, 42, 45). In contrast, characterization of [FeFe] hydrogenase maturation has been limited by a lack of knowledge of the proteins functioning in the biosynthesis of the catalytic H cluster. Recently, we identified two genes in *Chlamydomonas reinhardtii*, *hydEF* and *hydG*, which encode proteins required for [FeFe] hydrogenase activity by functioning in H cluster biosynthesis and enzyme maturation (40). Both proteins contain conserved motifs found in radical S-adenosylmethionine (SAM)-binding proteins, suggesting that biosynthesis of the H cluster during [FeFe] hydrogenase maturation involves radical-SAM-dependent mechanisms. The radical-SAM domains in both HydE and HydG contain the conserved motif C$_x$C$_x$C$_x$C, with additional motifs in the C-terminal ends that are characteristic of iron-sulfur cluster-binding sites (16). The HydF protein differs from HydE and HydG in that it belongs to the GTPase protein family (27). At the N-terminal end of HydF, there are motifs that match the conserved GTP-binding motifs, with a potential iron-sulfur cluster-binding motif present at the C-terminal end (27).

Although the biochemistry of [FeFe] hydrogenase maturation is an emerging field of investigation, the [FeFe] hydrogenases from several organisms have been extensively investigated by biochemical, spectroscopic, and structural methods (reviewed in reference 1). As a result of these investigations, a number of the characteristics of the H cluster and catalytic site have been elucidated. The H cluster is arranged as a unique [2Fe] center connected by a conserved cysteine to a [4Fe4S] cubane cluster (1, 35, 38). The iron atoms of the [2Fe] center are bridged by a dithio moiety which, based on a combination of structural and theoretical studies, is interpreted as either a propandithiol or a di(thiomethyl)amine (13, 34, 35, 38). Other features of the [2Fe] center are asymmetric CN and CO ligand pairs at each iron, with an additional CO bridging the two iron atoms (35, 38, 39). Thus, the HydF and HydG proteins are thought to function together in a series of undefined steps in synthesis and insertion of the chemically complex H cluster at the [FeFe] hydrogenase catalytic site during maturation.

As part of our previous report, we demonstrated that anaerobic coexpression of the *C. reinhardtii* [FeFe] hydrogenase maturation and structural genes in *Escherichia coli* resulted in the biosynthesis of an active [FeFe] hydrogenase (40). However, the *C. reinhardtii* *hydEF* and *hydG* clones were unstable and susceptible to rearrangements in *E. coli*, a problem that...
hindered investigations of the maturation process. In this study, we have developed a more efficient biosynthetic system by expression cloning of the hydE, hydF, and hydG homologues from *Clostridium acetobutylicum*. Anaerobic coexpression in *E. coli* of *C. acetobutylicum* maturation proteins HydE, HydF, and HydG with the [FeFe] hydrogenase from *C. acetobutylicum, Clostridium pasteurianum*, or *C. reinhardtii* resulted in the biosynthesis of a catalytically active enzyme. The stability of the *C. acetobutylicum* expression clones allowed us to initiate functional studies of HydE, HydF, and HydG in [FeFe] hydrogenase maturation. In addition, we found that catalytic-site maturation of structurally complex enzymes could be achieved in the presence of only the catalytic domain. Together, these results support the conclusion that fully functional HydE, HydF, and HydG maturation proteins and the [FeFe] hydrogenase catalytic domain are the fundamental components of the biosynthesis of active [FeFe] hydrogenases.

**MATERIALS AND METHODS**

Expression cloning of *C. acetobutylicum* HydA, HydE, HydF, and HydG. The *C. acetobutylicum* hyd genes were isolated from purified genomic DNA (strain ATCC 824) by PCR amplification. Gene-specific primers were based on the known sequence of *hydE* (GenBank accession no. AAC70723) (18, 47) and the sequences of *hydF* (GenBank accession no. CAC1631), *hydF* (GenBank accession no. CAC1651), *hydG* (GenBank accession no. CAC1356), and *hydB* (GenBank accession no. CAC3230) identified by BLASTn homology searches of the *C. acetobutylicum* genome (36) at the National Center for Biotechnology Information by using the *C. reinhardtii* hydE, hydF, and hydA2 peptide sequences (40). Gene-specific primers were designed to match the ends of each *hyd* gene (IDT Technologies) and also to contain a suitable restriction site for expression cloning. Approximately 20 ng of genomic DNA was digested overnight with BamHI and 200 ng was used as a template for PCR amplifications performed with KOD polymerase (Novagen). PCR fragments were gel purified, digested overnight with restriction enzymes, and subcloned into the dual multiple cloning sites (MCS) of either plasmid pCDFDuet-1 (Novagen) (hydF and hydG) or plasmid pETDuet-1 (Novagen) (hydA or hydB) to form pCaFG or plasmid pETDuet-1 (Novagen). The StrepII tag sequence (WSHPQFEK) was added to the 5′-end oligonucleotides designed with 5′-end nested primers (QIAGEN). Gene fragments were gel purified, digested overnight with restriction enzymes, and subcloned into the dual multiple cloning sites (MCS) of either plasmid pCDFDuet-1 (Novagen) (hydF and hydG) to form pCaFG or plasmid pETDuet-1 (Novagen) (hydA or hydB or hydG) to form pCaAE and pCaBE. The StrepII tag sequence WSHPOFEK was added to the C-terminal end of [FeFe] hydrogenase structural genes *hydA* and *hydB* during PCR amplification. The sequence and reading frame of each gene were confirmed by DNA sequencing (Davis Sequencing, LLC).

Expression cloning of other [FeFe] hydrogenase genes. The [FeFe] hydrogenase structural genes from *C. reinhardtii* and *C. pasteurianum* were cloned from purified genomic DNA as follows. To clone *C. pasteurianum* hyd (32), strain ATCC 6013 was cultured anaerobically on Clostridia Differential Broth (Sigma) with anhydrous sodium sulfate as the energy source. The 1-liter culture was grown at 37°C to a D_{570} of 0.5, and used to inoculate 1 liter of M63 (without Fe-citrate). The 1-liter culture was grown at 37°C to an OD_{590} of 0.5. A 1 mL solution of Fe-citrate was added to a final concentration of 100 μM, and the cultures were incubated for an additional 10 min at 37°C. IPTG (1.5 mM) was added, and the cultures were shaken at 100 rpm for 1 h at room temperature. Following the initial induction period, the cultures were transferred to a sealed 1-liter flask and sparged with argon at room temperature overnight to induce biosynthesis of [FeFe] hydrogenase.

Expression of [FeFe] hydrogenases for affinity purification was performed in minimal medium. Transformed cells were cultured overnight in 5 ml of M63 (49) supplemented with 0.5% glucose, 0.4% casein-hydrolysate, 100 μM Fe-citrate, 300-μg/ml ampicillin, and 50-μg/ml streptomycin. Cultures grown overnight were diluted 1:50 to 25 ml of fresh medium, grown at 37°C until the OD_{590} reached 0.5, and used to inoculate 1 liter of M63 (without Fe-citrate). The 1-liter culture was grown at 37°C to an OD_{590} of 0.5. A 1 mL solution of Fe-citrate was added to a final concentration of 100 μM, and the cultures were incubated for an additional 10 min at 37°C. IPTG (1.5 mM) was added, and the cultures were shaken at 100 rpm for 1 h at room temperature. Following the initial induction period, the cultures were transferred to a sealed 1-liter flask and sparged with argon at room temperature overnight to induce biosynthesis of [FeFe] hydrogenase.

Purification of recombinant, StrepII-tagged [FeFe] hydrogenases. Purification steps were performed under anaerobic conditions. Cells expressing StrepII-tagged [FeFe] hydrogenases were collected by centrifugation at 6,000 × g for 10 min, and the cell pellet was suspended in break buffer (BB) (150 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM diithiothreitol, 1 mM Na-dithionite, 100 μM phenylmethylsulfonyl fluoride, 5% glycerol) and broken in a French press. Avi-din was added at 3 nM to block binding of biotin and biotinylated proteins. The disrupted cell suspensions were centrifuged at 19,000 × g for 30 min to pellet cell debris. The clarified crude extracts were passed over a StreptTacin-Septharose (IBA, Göttingen, Germany) affinity column pre-equilibrated with BB. Columns were washed with 3 to 5 column volumes of ice-cold BB, and the StreptII-tagged [FeFe] hydrogenases were eluted in BB containing 2.5 mM desthiobiotin.

**SDS-PAGE and Western blotting.** For sodium dodecyl sulfate (SDS)-poly- acrylamide gel electrophoresis (PAGE), protein samples were diluted in 4× SDS-PAGE loading buffer (Novagen), boiled for 10 min, and cooled on ice. Samples were loaded onto a 12% SDS gel and run at 45 mA for 2 h. Following electrophoresis, proteins were blotted onto polyvinylidene difluoride membranes and detected with a StreptTacin-alkaline phosphatase conjugate detection kit (IBA).

**[FeFe] hydrogenase activity assays.** Activities of purified [FeFe] hydrogenases or of whole-cell extracts were routinely measured as the evolution of H₂ gas from reduced methyl viologen (MV). Activity assays of whole cells were performed with argon-charged 13.5-mL sealed serum vials that contained 1 mL of an anaerobically prepared 2× whole-cell reaction buffer (50 mM potassium phosphate, pH 7; 10 mM MV; 20 mM sodium dithionite; 6 mM NaOH; 0.2% Triton X-100) and 1 mL of cells. Assays of purified enzymes were performed with aliquots (5 to 50 μl) diluted to 1 ml in anaerobically prepared BB in an argon-flushed vial that also contained 1 mL of an anaerobically prepared 2× enzyme reaction buffer (50 mM potassium phosphate, pH 7; 10 mM MV; 20 mM sodium dithionite; 6 mM NaOH). All reaction mixtures were incubated at 37°C. After incubation, 400 μl of headspace gas was removed with a gas-tight syringe and H₂ levels were measured by gas chromatography (Hewlett Packard 5820). H₂ oxidation activities of purified enzymes were measured spectrophotometrically in a sealed cuvette containing an H₂-saturated reaction buffer composed of 100 mM Tris-HCl (pH 8) and 1 mM MV. Purified enzyme was added, and the absorbance at 604 nm was monitored. An extinction coefficient for reduced MV of 13.6 mM⁻¹ cm⁻¹ was used to convert absorbance values to micromoles of H₂ oxidized per minute. Measurements of the Kₘ of algal HydA2 [FeFe] hydrogenase with reduced ferredoxin were performed with commercially prepared spinach ferredoxin (Sigma). Individual reaction mixtures contained ~100 μg of affinity-purified HydA2 and 100 μM ferredoxin in 1 mL of ferredoxin reaction buffer (15 mM Na-dithionite, 50 mM Tris-HCl, pH 7). Evolution of H₂ gas was measured by gas chromatography.
**RESULTS**

Expression and biosynthesis of *C. acetobutylicum* [FeFe] hydrogenase HydA in *E. coli*. We previously reported on the expression of active [FeFe] hydrogenase in *E. coli* cells that had been transformed with T7 expression plasmids encoding the genes for *C. reinhardtii* maturation proteins HydEF and HydG and the StrepII-tagged *C. acetobutylicum* hydrogenase HydA1 (40). Although the purified algal [FeFe] hydrogenase was active, the instability of the expression plasmids made the transformants difficult to propagate, which resulted in low *C. reinhardtii* HydA1 expression levels. The DNA compositions of algal genes are highly GC biased at 64% overall and 90% at the third codon position (48). To address codon bias effects on gene stability and expression, we searched the sequenced genomes of various anaerobic microbes for homologues of hydEF and hydG as alternatives to the algal genes. The genome of *C. acetobutylicum* (36) was found to possess hydE, hydF, and hydG homologues, in agreement with previous reports on the characterization of a soluble, monomeric [FeFe] hydrogenase (*C. acetobutylicum* HydA) in this organism (18, 47). Unlike the high GC content of the *C. reinhardtii* hydEF (70%) and hydG (65%) genes, the *C. acetobutylicum* genes were more AT rich (GC contents: hydE, 32%; hydF, 33%; hydG, 35%) and thus were expected to be more stable and better expressed in *E. coli*. The *C. acetobutylicum* hydE, hydF, and hydG genes were PCR amplified, and the products were cloned into a set of T7 expression plasmids together with the *C. acetobutylicum* hydA [FeFe] hydrogenase gene modified to encode StrepII-tagged *C. acetobutylicum* HydA (Fig. 1). Plasmids that harbored a complete set of *C. acetobutylicum* maturation and structural genes were transformed into *E. coli* strain BL21(DE3) for IPTG-inducible T7 expression. Compared to the plasmid-encoded *C. reinhardtii* hydEF and hydG genes, which were observed to undergo rearrangements upon propagation in *E. coli* (unpublished results), the plasmid-encoded *C. acetobutylicum* hydE, hydF, and hydG genes did not exhibit any sequence alterations. The higher stability of the *C. acetobutylicum* genes resulted in greater numbers of transformed cells and higher growth rates under expression conditions (data not shown).

When *E. coli* is cultured anaerobically in the absence of fermentable sugars, the maturation of endogenous [NiFe] hydrogenases Hyd1, Hyd2, and Hyd3 is limited by the lack of formate. Formate is a fermentative metabolite required for the transcriptional activation of the *hyp* and *hyc* operons encoding [NiFe] hydrogenase maturation proteins and the Hyd3 enzyme, respectively (24, 30). As a result of the lack of *hyp* expression, anaerobic growth of *E. coli* in the absence of formate results in basal levels of all [NiFe] hydrogenase activities in whole-cell extracts, as shown in Table 1. The basal [NiFe] hydrogenase activity under these growth conditions allows for study of the maturation and biosynthesis of recombinantly overexpressed [FeFe] hydrogenases. As shown in Table 1, the extracts of anaerobically grown *E. coli* cells expressing the *C. acetobutylicum* maturation system with the StrepII-tagged *C. acetobutylicum* HydA structural protein exhibited in vitro H₂ evolution activities that were 275-fold greater than the activities in extracts of untransformed cells. The elevated hydrogenase activities are directly attributable to induced coexpression of the plasmid-encoded *C. acetobutylicum* maturation and structural genes, resulting in accumulation of maturation proteins and the biosynthesis of StrepII-tagged *C. acetobutylicum* HydA [FeFe] hydrogenase.

The *C. acetobutylicum* maturation system developed in this study produced milligram-per-liter concentrations of StrepII-tagged *C. acetobutylicum* HydA, whereas our previ-

<table>
<thead>
<tr>
<th>Organism</th>
<th>[FeFe] hydrogenase</th>
<th>Whole-cell extracta</th>
<th>Affinity purifiedb</th>
</tr>
</thead>
<tbody>
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<td><em>E. coli</em></td>
<td>0.35c</td>
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<td>ND</td>
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<tr>
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<td>HydA</td>
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<td>150</td>
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<tr>
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<tr>
<td><em>C. acetobutylicum</em></td>
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<td>75.2</td>
</tr>
<tr>
<td></td>
<td>HydA</td>
<td>6</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>HydB</td>
<td>13</td>
<td>8.6</td>
</tr>
<tr>
<td><em>C. pasteurianum</em></td>
<td>HydA</td>
<td>150</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>HydA</td>
<td>15</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Whole cells solubilized with 0.1% Triton X-100.

b Whole-cell activity in the absence of [FeFe] hydrogenase structural and maturation proteins.

ND, not determined.
ous *C. reinhardtii* maturation system produced only microgram-per-liter concentrations of StrepII-tagged *C. reinhardtii* HydA (40). As shown in Table 1, the H₂ evolution activities of extracts of whole cells expressing either StrepII-tagged *C. acetobutylicum* HydA or *C. pasteurianum* HydA were similar in value. The specific activity of affinity-purified StrepII-tagged *C. acetobutylicum* HydA was 75 μmol of H₂ evolved mg⁻¹ min⁻¹. This specific activity value is 7.5-fold higher than previously reported values (17) for the StrepII-tagged enzyme recombinantly expressed and purified from anaerobically grown *C. acetobutylicum*.

Whereas the StrepII-tagged *C. acetobutylicum* HydA purified from our recombinant system has an H₂ evolution specific activity from reduced MV that is 53-fold lower than that of *C. pasteurianum* HydA purified from *C. pasteurianum* (3), the amino acid alignment in Fig. 2 shows that *C. acetobutylicum* HydA and *C. pasteurianum* HydA have high sequence similarity and 70% identity (18, 47). Moreover, the enzymes have nearly identical amino acid compositions within their H cluster motifs. The *C. acetobutylicum* HydA sequence also displays a complement of accessory iron-sulfur cluster (F cluster) motifs similar to that of the N-terminal domain of *C. pasteurianum* HydA (2, 3, 38). The small number of amino acid differences between the catalytic sites of the two enzymes, however, might contribute in some manner to the difference in the specific activities of the two enzymes.

**Expression and biosynthesis of *C. acetobutylicum* [FeFe] hydrogenase HydB in *E. coli***. In addition to hydA, the *C. acetobutylicum* genome possesses a second gene, hydB, coding for a second, putative [FeFe] hydrogenase. Unlike *C. acetobutylicum* HydA, the *C. acetobutylicum* HydB amino acid sequence, shown in Fig. 2, has low sequence similarity to *C. pasteurianum*.
HydA, and the two are only 18.6% identical overall. The C. acetobutylicum HydA and the two are only 18.6% identical overall. The C. acetobutylicum HydA underlined (Fig. 2). The N-terminal end of the C. acetobutylicum HydA underlined (Fig. 2). The N-terminal end of the C. acetobutylicum HydA but retain the strictly conserved cysteines required for H cluster coordination (38). The sequences in C. acetobutylicum HydB that are similar to motifs H1C, H1C, and H3C are SCNCPS, GPC, and MGCVGSGC, respectively, with the cysteines that coordinate the H cluster in C. pasteurianum HydA underlined (Fig. 2). The N-terminal end of the C. acetobutylicum HydB sequence possesses a single motif with similarity to the conserved, catalytic-site-proximal, F cluster-binding motif of C. pasteurianum HydA (identified as F4 in Fig. 2). Upstream of this F cluster motif in C. acetobutylicum HydB are six additional cysteines, some of which might function in the coordination of a second F cluster. The presence of two F cluster motifs in C. acetobutylicum HydB would be similar to the F cluster arrangements of [FeFe] hydrogenase II from C. pasteurianum (HydB) (2, 3) and the periplasmic [FeFe] hydrogenase of Desulfovibrio desulfuricans (35).

To determine if C. acetobutylicum HydB is a functional hydrogenase, a StrepII-tagged derivative was coexpressed with the Hyd maturation proteins and affinity purified. As shown in Fig. 2 and Table 1, StrepII-tagged C. acetobutylicum HydB was biosynthesized as an active hydrogenase. Purified, StrepII-tagged C. acetobutylicum HydB catalyzed H2 evolution from reduced MV at a rate of 8.6 µmol of H2 min−1 mg−1, a value approximately ninefold lower than the specific activity of purified, StrepII-tagged C. acetobutylicum HydA (Table 1). H2 oxidation coupled to MV reduction by StrepII-tagged C. acetobutylicum HydB was measured at a specific activity of 19 µmol of H2 oxidized min−1 mg−1, about twofold higher than the H2 evolution rate. The ratio of H2 oxidation to H2 evolution with MV as the electron carrier for StrepII-tagged HydB purified from C. acetobutylicum is 2, whereas HydB (CpII) purified from C. pasteurianum was previously shown to have a ratio of 15 (2).

**Biosynthesis of heterologous [FeFe] hydrogenases by the C. acetobutylicum maturation proteins.** Our interest in the biochemical and structural properties of the [FeFe] hydrogenases found in the green alga C. reinhardtii prompted a test of the capability of the C. acetobutylicum maturation proteins to biosynthesize the C. reinhardtii [FeFe] hydrogenases HydA1 and HydA2 in E. coli. A striking characteristic of the algal [FeFe] hydrogenase peptide sequences is the lack of F cluster domains auxiliary to the highly conserved H cluster/catalytic domain (Fig. 4) (14, 15). This reduced structural complexity classifies the algal hydrogenases as the simplest yet characterized (14, 50).

In C. reinhardtii, the HydA1 [FeFe] hydrogenase undergoes N-terminal processing as a result of translocation from the cytoplasm to the chloroplast stroma (22). Similar to C. reinhardtii HydA1, the N-terminal sequence of C. reinhardtii HydA2 also possesses signal sequence characteristics (15) with a predicted cleavage site near amino acid position 61. For expression in E. coli, a truncated hydA2 gene was cloned into expression plasmid pCaE, which created an N terminus at position 62 that corresponds to the predicted processed product. As shown in Table 1 and Fig. 3, the mature forms of StrepII-tagged C. reinhardtii HydA1 and HydA2 were both biosynthesized as active enzymes in E. coli. Following affinity purification, the typical yields of these enzymes ranged from 0.8 to 1.0 mg/liter of culture. The specific activities of purified StrepII-tagged C. reinhardtii HydA1 and HydA2 were 150 and 116 µmol of H2 evolved mg−1 min−1, respectively. This measured activity for StrepII-tagged C. reinhardtii HydA1 purified from our E. coli expression system was five- to sixfold lower than the previously published activities of this enzyme purified from recombinant (17) or native (14) sources (see Discussion).

![F-cluster Binding Domain](image-url) ![Catalytic Domain](image-url)

**FIG. 4.** Schematic representation of aligned sequences of the [FeFe] hydrogenases used in this study (Ca, C. acetobutylicum; Cp, C. pasteurianum; Cr, C. reinhardtii). The top diagram represents the relative locations of the conserved F cluster-binding domain (cross-hatched bar) and the catalytic domain (gray-shaded bar) with H cluster-binding motifs HC1, HC2, and HC3 (dark bars) found in soluble [FeFe] hydrogenases (50). The arrowheads indicate the N termini of conserved F cluster-binding domains deleted in C. acetobutylicum and C. pasteurianum HydAΔN constructs.
It has been established that under anaerobic conditions, *C. reinhardtii* utilizes reduced [2Fe2S] ferredoxin as an electron donor to [FeFe] hydrogenase for in vivo H\(_2\) evolution (6, 44). Previous measurements of H\(_2\) evolution kinetics with partially purified *C. reinhardtii* hydrogenases and reduced *C. reinhardtii* [2Fe2S] ferredoxin showed a \(K_m\) of 10 \(\mu\)M (43). The \(K_m\) of reduced spinach [2Fe2S] ferredoxin for purified *C. reinhardtii* HydA2 in this study was measured at 31 \(\mu\)M. This value is similar to the previous reported value of 35 \(\mu\)M for purified *C. reinhardtii* HydA1 and reduced spinach ferredoxin (23) and suggests that *C. reinhardtii* HydA2 is capable of catalyzing in vivo H\(_2\) evolution and/or H\(_2\) oxidation in *C. reinhardtii* under anaerobic conditions.

**Biosynthesis of N-terminally deleted *C. acetobutylicum* HydA and *C. pasteurianum* HydA.** The ability of the *C. acetobutylicum* maturation proteins to biosynthesize the algal enzymes suggests that the process of catalytic-site maturation is independent of the structural contributions of the F cluster-binding domain. This observation was tested by deleting the N-terminal F cluster-binding domains of *C. acetobutylicum* hydA and *C. pasteurianum* hydA (illustrated in Fig. 4) and expressing the StrepII-tagged, truncated structural proteins in cells coexpressing the *C. acetobutylicum* maturation proteins. As shown in Table 1, extracts of anaerobically grown cells that expressed either StrepII-tagged *C. acetobutylicum* HydA\(\Delta\)N or *C. pasteurianum* HydA\(\Delta\)N showed similar \(H_2\) evolution activities from reduced MV. Affinity purification of StrepII-tagged *C. acetobutylicum* HydA\(\Delta\)N yielded ~0.5 to 1 mg per liter of culture, with \(H_2\) evolution activities that were ~2.4-fold lower than those of structurally complete *C. acetobutylicum* HydA. In the absence of the N-terminal F cluster domain, StrepII-tagged *C. acetobutylicum* HydA\(\Delta\)N was unable to catalyze sufficient \(H_2\) evolution from reduced clostridial ferredoxin (data not shown).

**Site-directed mutagenesis of conserved motifs in HydF.** The maturation protein HydF differs from HydE and HydG in that it lacks a conserved radical-SAM-binding motif (40). Rather, HydF is composed of an N-terminal domain that is homologous to the GTPase protein family and a C-terminal domain that contains a putative iron-sulfur cluster-binding motif. The GTPase domains of the HydF proteins from *C. reinhardtii* and *C. acetobutylicum* each possess motifs, shown in Table 2, that are homologous to the glycine-rich P-loop, magnesium-binding, and GTP-specific distal motifs characteristic of the GTPase protein family (27). The observation that all of the HydF homologues identified to date share an N-terminal GTPase domain suggests that GTPase activity might be required for the biosynthesis and maturation of [FeFe] hydrogenase. To test the requirement for an intact GTP-binding P-loop motif, GxxNxGKS, single-site mutations of conserved P-loop residues G19, G24, and S26 in *C. acetobutylicum* HydF were made and tested for maturation activity. The results in Table 3 show that the G19A, G24A, and S26A mutations all resulted in severe impairment of [FeFe] hydrogenase maturation under anaerobic inducing conditions. The impaired \(H_2\) evolution activities in extracts of cells that coexpressed mutant HydF variants together with HydE, HydG, and StrepII-tagged *C. acetobutylicum* HydA were similar to the activities of cell extracts expressing HydE, HydG, and *C. acetobutylicum* HydA in the absence of HydF.

**Site-directed mutagenesis of conserved motifs in HydE and HydG.** The HydE and HydG maturation proteins belong to the radical-SAM superfamily. These proteins both contain the signature C\(_x\)C\(_x\)C motif that functions in the binding of a redox-active [4Fe4S] cluster and a molecule of SAM (Table 4) (16). Recently, reconstituted forms of HydE and HydG from *Thermotoga maritima* have been shown to reductively cleave SAM, confirming their identification as radical SAM proteins (46). As shown in Table 3, single-site cysteine-to-serine mutations in the C\(_x\)C\(_x\)C SAM-binding motifs in either HydG or HydE led to a 300- to 1,000-fold reduction, respectively, of \(H_2\) evolution activities in extracts of transformed cells expressing StrepII-tagged *C. acetobutylicum* HydA. The background levels of C.

### Table 2. Sequences matching conserved motifs in maturation protein HydF

<table>
<thead>
<tr>
<th>Motif*</th>
<th>Conserved sequencea,b</th>
<th>Organism</th>
<th>Amino acid alignmentc</th>
<th>HydF consensusb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walker A P loop</td>
<td>GxxxxGKS/T</td>
<td><em>C. acetobutylicum</em></td>
<td>GTIN/GKS</td>
<td>CxxxxGKS</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. reinhardtii</em></td>
<td>GVS/GNS</td>
<td></td>
</tr>
<tr>
<td>Walker B Mg(^{2+}) binding</td>
<td>hhhhDxxG</td>
<td><em>C. acetobutylicum</em></td>
<td>VML/DTAG</td>
<td>hxLhDTAG</td>
</tr>
<tr>
<td>Distal, GTP specificity</td>
<td>N/TKxD</td>
<td><em>C. acetobutylicum</em></td>
<td>NK/D</td>
<td>NKxD</td>
</tr>
<tr>
<td>FeS cluster binding</td>
<td>CxxH(_{46-53})CxxC</td>
<td><em>C. acetobutylicum</em></td>
<td>C/TH(_{46})HC/AGC</td>
<td>CxxH(_{46-53})HCGGC</td>
</tr>
</tbody>
</table>

\(\text{a GTP-binding motifs (13).}
\(\text{b Lowercase letters: x, any amino acid; h, hydrophobic.}
\(\text{c Boldface, identical; italic, similar.}

* a

* b
HydA synthesized from mutant HydE and HydG maturation systems were similar to the levels of cells coexpressing incomplete maturation systems of HydF and HydG or HydE and HydF, respectively.

In addition to the cysteine-rich radical-SAM motif, the HydG proteins exhibit a second conserved cysteine motif, Cx2Cx2C. Based on the arrangement of cysteines and biochemical investigations of reconstituted T. maritima HydG, the

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**TABLE 4. Sequences matching conserved motifs in maturation proteins HydE and HydG**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Motif&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conserved sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Organism</th>
<th>Amino acid alignment&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Consensus&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HydE</td>
<td>SAM binding</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>C. acetobutylicum</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. reinhardtii</td>
<td>CRNCMYC</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CNDGNYC</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
</tr>
<tr>
<td>HydG</td>
<td>SAM binding</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>C. acetobutylicum</td>
<td>Cy&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. reinhardtii</td>
<td>CYNCVYC</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
</tr>
<tr>
<td></td>
<td>FeS cluster binding</td>
<td>Cx&lt;sub&gt;3&lt;/sub&gt;Cx&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>C. acetobutylicum</td>
<td>CTACE&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>CTACE&lt;sub&gt;3&lt;/sub&gt;C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. reinhardtii</td>
<td>CTACE&lt;sub&gt;3&lt;/sub&gt;C</td>
<td>CTACE&lt;sub&gt;3&lt;/sub&gt;C</td>
</tr>
</tbody>
</table>

<sup>a</sup> SAM-binding motifs (12).

<sup>b</sup> Lowercase x, any amino acid.

<sup>c</sup> Underlining indicates identical amino acids.
second motif is suggested to coordinate an additional iron-sulfur cluster (Table 4) (40, 46). A similar motif is also featured in the radical-SAM-dependent proteins BioB and lipoyl synthase (LS) from *E. coli* and MoaA from *Staphylococcus aureus*, which all possess a second iron-sulfur cluster (7, 11, 19, 20). Interestingly, in two of these proteins, BioB and LS, the extra iron-sulfur cluster is proposed to be involved in the incorporation of sulfide into organic precursors during cofactor biosynthesis (7, 11). As shown in Table 3, single cysteine-to-serine mutations of the Cx2Cx22C motif resulted in severe impairment of the ability of transformed cells to biosynthesize active levels of StrepII-tagged *C. acetobutylicum* HydA.

**DISCUSSION**

The early (52) and more recent (4, 40) attempts at recombinant expression of [FeFe] hydrogenases in non-native systems, including *E. coli*, have shown some limited success. Plasmid clones of the *D. vulgaris* [FeFe] hydrogenase expressed in *E. coli* resulted in the synthesis of enzymes that possessed only the F clusters without the catalytic H cluster (52). Recombinant expression of *C. pasteurianum* hydA in the cyanobacterium *Synechococcus* sp. strain PCC7942 resulted in whole cells and cell extracts with increased hydrogenase activities (4). However, the absence of [FeFe] hydrogenase structural and maturation proteins in *Synechococcus* led the authors to speculate that the native [NiFe] hydrogenase maturation proteins were responsible for clostridial [FeFe] hydrogenase biosynthesis (4). This possibility seems unlikely, given our results demonstrating that a complete and functional [FeFe] hydrogenase maturation system composed of the HydE, HydF, and HydG proteins is a basic requirement to achieve biosynthesis of an active [FeFe] hydrogenase. Nevertheless, based on our earlier results (40) and those presented here, the cotransformation of [FeFe] hydrogenase structural and maturation genes in microbes that lack a native [FeFe] hydrogenase can clearly result in the biosynthesis of active hydrogenases.

Recently, it was demonstrated that [FeFe] hydrogenases from the green alga species *C. reinhardtii* and *Scenedesmus obliquus* could be recombinantly expressed and biosynthesized in *C. acetobutylicum* (17). The authors reported a specific activity of purified, StrepII-tagged *C. reinhardtii* HydA1 that was about fivefold higher than those reported here for the same enzyme purified in our *E. coli* recombinant expression system. However, the H₂ evolution specific activities for the StrepII-tagged *C. acetobutylicum* HydA protein we purified from our *E. coli* expression system were 7.5-fold greater than the value reported for the same enzyme purified from the native recombinant system (17). The differences in the specific activity levels of different [FeFe] hydrogenases recombinantly produced from *E. coli* versus *C. acetobutylicum* might be due to differences in expression conditions, levels of available substrates, or levels of other auxiliary factors (e.g., proteins, cofactors) to optimize maturation. Moreover, the *E. coli* system described here utilizes T7 expression of the genes encoding [FeFe] hydrogenase maturation and structural proteins. The resulting high expression levels of Hyd proteins might cause apo forms to accumulate due to the elevated demand for iron-sulfur clusters. Previous studies have shown that iscSU4 expression is required for the biosynthesis of a variety of radical-SAM- and iron-sulfur cluster-containing proteins in *E. coli* (11, 19, 20, 33), and elevated levels of iscSU4 expression might improve the levels of [FeFe] hydrogenase biosynthesis in *E. coli*. In the case of StrepII-tagged *C. acetobutylicum* HydB, which has low sequence similarity to *C. acetobutylicum* HydA (~19%), maturation to an active hydrogenase by the *C. acetobutylicum* Hyd proteins suggests that it might also possess a similar H cluster. However, determination of its physiological role in *C. acetobutylicum* in either H₂ oxidation or H₂ evolution requires further in vivo studies.

The new recombinant [FeFe] hydrogenase expression system developed in this study has facilitated initial investigations into the structure and function of maturation proteins for H cluster biosynthesis and for [FeFe] hydrogenase maturation. Consistent with recent results showing that *T. maritima* HydE and HydG possess SAM cleavage activity (40), mutations of conserved cysteines in the SAM-binding motifs of either *C. acetobutylicum* HydE or HydG resulted in defective maturation of *C. acetobutylicum* HydA [FeFe] hydrogenase. The fact that, in *C. reinhardtii*, HydE and HydF are linked to form a single HydEF peptide (40) suggests that, mechanistically, HydE and HydF are likely to form a functional complex during H cluster biosynthesis. The additional C-terminal cysteines of HydF also required for maturation might function to coordinate an additional iron-sulfur cluster involved in H cluster biosynthesis. Interestingly, radical-SAM motifs are in HydE and HydG, and in most cases both proteins appear to possess a second, additional, iron-sulfur cluster (40, 46). Both the radical-SAM and iron-sulfur cluster motifs in HydG were shown by mutational analysis in this study to be essential for [FeFe] hydrogenase maturation. The involvement of two radical-SAM-dependent proteins in H cluster biosynthesis likely reflects the unusual biochemistry required to synthesize an iron-sulfur cluster that possesses CN, CO, and dithio ligands.

Biosynthesis and maturation of the nickel enzymes urease, CO-dehydrogenase, and [NiFe] hydrogenase, as well as the FeMo cofactor of nitorgenase, involve nucleotide-dependent steps mediated by a specific nucleotide-binding protein (25, 26, 31, 41). The nucleotide-dependent steps in the maturation of [NiFe] hydrogenases involve GTP hydrolysis coupled to nickel donation (31) and ATP hydrolysis coupled to the synthesis of CN ligands to iron (9). In the case of the [FeFe] hydrogenase maturation protein HydF, mutational analysis has shown that an intact putative GTP-binding P-loop motif, GxxNxxGKS, was essential to achieve biosynthesis of [FeFe] hydrogenase. How the HydE and HydG SAM cleavage activities function in combination with HydF nucleotide hydrolysis to achieve H cluster biosynthesis and [FeFe] hydrogenase maturation remains to be elucidated.

Our results clearly show that H cluster biosynthesis is a highly conserved process. Together with recent structural data on the [FeFe] hydrogenases Cpl (called *C. pasteurianum* HydA in this study) and DdH (35, 38), our work supports earlier observations (1) that various [FeFe] hydrogenases might possess very similar H clusters. The biosynthesis of compositionally diverse structural proteins (i.e., algal versus bacterial) by a single maturation system corroborates the domain conservation proposed for this family of enzymes (50). Presumably, an [FeFe] hydrogenase precursor(s) might have evolved to acquire or lose accessory cluster domains, with coevolution of the
maturation process to maintain biosynthetic efficiency. In this regard, perhaps the reduced specific activities observed for truncated [FeFe] hydrogenases might indicate that the F cluster domains of more structurally complex enzymes contribute in some manner to efficient enzyme maturation.

The efforts to develop biological alternatives to fossil fuels have helped stimulate an ongoing interest in the use of microorganisms as production sources for a number of energy carriers. The physiology of H2-producing organisms and the hydrogenases that mediate H2 metabolism have been intensely studied for use as large-scale H2 sources. A greater understanding of how [FeFe] hydrogenases are biosynthesized and how their unique structures contribute to biochemical and metabolic function will assist the continued development of both biological and biologically inspired H2 production systems.

ACKNOWLEDGMENTS

We acknowledge and thank the members of the National Renewable Energy Laboratory Biological Sciences Team for stimulating discussions and helpful ideas.


REFERENCES


