HydF as a scaffold protein in [FeFe] hydrogenase H-cluster biosynthesis

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Abstract In an effort to determine the specific protein component(s) responsible for in vitro activation of the [FeFe] hydrogenase (HydA), the individual maturation proteins HydE, HydF, and HydG from \textit{Clostridium acetobutylicum} were purified from heterologous expressions in \textit{Escherichia coli}. Our results demonstrate that HydF isolated from a strain expressing all three maturation proteins is sufficient to confer hydrogenase activity to purified inactive heterologously expressed HydA (expressed in the absence of HydE, HydF, and HydG). These results represent the first in vitro maturation of [FeFe] hydrogenase with purified proteins, and suggest that HydF functions as a scaffold upon which an H-cluster intermediate is synthesized.

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1. Introduction

The [FeFe] and [NiFe] hydrogenases catalyze hydrogen oxidation and proton reduction and function either to couple hydrogen oxidation to energy yielding processes or to regenerate reduced electron carriers accumulated during fermentation [1]. Although phylogenetically unrelated, these enzymes share biologically unique sites containing iron with both cyanide and carbon monoxide ligands [2]. These strong field ligands presumably function to stabilize low oxidation states of iron. The [NiFe] hydrogenase active site consists of an iron atom bridged by one carbon monoxide and two cyanide ligands [2]. These strong ligands presumably function to stabilize low oxidation states of iron. The [NiFe] hydrogenase active site consists of an iron atom bridged by one carbon monoxide and two cyanide ligands bridged to a nickel atom via two cysteine thiolates. The [FeFe] hydrogenase active site consists of the “H-cluster” which exists as a [4Fe4S] cluster coordinated by carbon monoxide and cyanide ligands. In addition to these ligands, the irons of the subcluster are bridged by a non-protein dithiolate linkage.

While progress has been made in defining the role of specific gene products and identifying the precursors of the non-protein ligands in the [NiFe] hydrogenases [3] relatively little is known about maturation of the “H-cluster” of the [FeFe] hydrogenases. Recent studies involving the analysis of mutants of \textit{Chlamydomonas reinhardtii} defective in hydrogen production have revealed that products of the \textit{hydEF} and \textit{hydG} genes are required for the accumulation of active [FeFe] hydrogenase, and that \textit{hydE}, \textit{hydF}, and \textit{hydG} are common to all organisms in which active [FeFe] hydrogenases are found [4]. Further studies have shown that the coexpression in \textit{Escherichia coli} of \textit{HydE}, \textit{HydF}, and \textit{HydG} from \textit{Clostridium acetobutylicum} with the HydA [FeFe] hydrogenase protein from a variety of microbial sources enables the formation of active [FeFe] hydrogenases [5]. Genome annotation indicates that HydE and HydG are members of the radical S-adenosylmethionine (AdoMet) enzyme family, and that HydF is likely to be a GTPase [4]. Preliminary biochemical characterization of heterologously expressed HydE, HydF, and HydG from \textit{Thermatoga maritima} corroborated the general functional inferences derived from genomic analysis and showed that all three proteins are capable of binding FeS clusters [6,7]. In our most recent work, we have shown that cell extracts of \textit{E. coli} in which all three Hyd proteins from \textit{C. acetobutylicum} are expressed simultaneously can activate heterologously expressed HydA [8]. The in vitro activation of HydA occurs only with extracts of \textit{E. coli} in which all three Hyd proteins are co-expressed and has been interpreted to indicate that a protein associated intermediate in cluster biosynthesis is generated under these conditions and readily transferred to the \textit{hydA} gene product to accomplish activation.

Following this development, we have pursued the identification of the activating component present in the cell extract. Purification of the three individual maturation proteins from strains in which all three Hyd proteins were expressed in concert revealed that HydF from this background (HydF\textsuperscript{EFG}) mediates [FeFe] hydrogenase activation, and HydF expressed in the absence of HydE and HydG (HydF\textsuperscript{E}) is not able to effect activation. The activation of HydA expressed in a genetic background devoid of HydE, HydF, and HydG (HydA\textsuperscript{EFG}) by HydF\textsuperscript{E} occurs in a manner consistent with the previously described in vitro activation of HydA\textsuperscript{EFG}, but does not
require the presence of HydE and HydG during the activation process.

2. Materials and methods

2.1. Cloning and heterologous expression of Hyd proteins

The hyd genes were PCR amplified by the Expanded Hi-Fidelity Plus PCR system (Roche) from purified C. acetobutylicum chromosomal DNA utilizing gene specific primers designed to contain suitable restriction sites allowing for the presence or absence of a 6x-histidine affinity tag (His6) and for cloning into the multiple cloning sites of Novagen Duet vectors (supplemental material). HydA from Clostridium pasteurianum was cloned as described previously [5] modified for the presence of an N-terminal 6x-histidine tag. Constructs were confirmed by DNA sequencing.

BL21 (DE3) cell lines were transformed with the appropriate plasmid constructs to create individual expression strains in which hyd gene products could be expressed singly or in tandem with or without 6x-histidine tags for affinity purification of a single protein. Transformation was followed by selection with appropriate antibiotics. Growth of the cell lines and protein expression was performed as described in the supplementary material.

2.2. Protein purification and UV/visible spectrophotometry

All purifications were performed by immobilized metal affinity chromatography (IMAC) using HisTrap HP Ni2+-affinity resins or columns (GE Healthcare) under strict anaerobic conditions at 4°C or at room temperature (see supplementary material) in a Coy anaerobic chamber (Coy Laboratories). Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard [9]. Iron content was evaluated spectrophotometrically [10]. For UV and visible absorption experiments, samples were transferred to an anaerobic cuvette within an MBraun glove box. Room temperature UV/vis absorption data were acquired using a Cary 6000 UV/vis near-IR spectrophotometer (Varian). Reduced samples were prepared by the addition of dithionite (2 mM final concentration). UV/vis spectra were collected at a data interval of 0.5 nm and a scan rate of 60 nm/min.

2.3. In vitro hydrogenase activation assay

Determination of the activity of a given Hyd maturation protein to activate HydA_EFG was tested by combining purified inactive HydA_EFG with the respective purified Hyd maturation protein, in addition to the assay reagents methyl viologen and dithionite. In this scheme, dithionite serves as an electron donor with methyl viologen acting as an electron conduit to HydA. Assay mixtures were prepared in a Coy anaerobic chamber at 25°C by mixing HydA_EFG (4.4 µg) with an excess of purified maturation protein (typically ~350 µg) in 3 ml sealable glass vials. Each 2 ml reaction was performed in 50 mM Hepes, pH 7.4, 500 mM NaCl in the presence of 20 mM sodium dithionite. The reactions were sealed, removed from the anaerobic chamber, and degassed to remove residual hydrogen from the headspace. Hydrogenase assays were performed at 25°C and initiated by the addition of oxidized methyl viologen (10 mM final concentration) and the production of H2 was measured using gas chromatography as described previously [8].

2.4. Investigating the stoichiometry of HydF_EFG activating HydA_EFG

Experiments directed at determining the maximal activation of HydA_EFG and the effects of increasing the amount of HydF_EFG were performed by adding increasing amounts of HydF_EFG (from 0.07 to 35 nmol) to a constant amount of HydA_EFG (0.07 nmol). Assays were performed as described above following a 20 minute incubation period and raw data were fit using linear regression to obtain hydrogen evolution rates. The quantity of HydA_EFG activated was estimated by comparison to the activity of native [FeFe] hydrogenase purified from C. pasteurianum (CpI). Although it was previously shown that the in vitro activation of hydrogenases from a variety of sources is possible [8], native CpI was chosen as a benchmark for hydrogenase activity in these studies because it is readily available and allows for reliable activity comparison. We did not utilize the activity of heterologously isolated CpI co-expressed with the requisite accessory proteins as an activity benchmark in these studies because previous work showed that such preparations do not exhibit the full native hydrogenase activity. Under the conditions described above the activity of native CpI was determined to be 304 µmol H2 min-1 mg-1 which was defined as 100% activity. In order to determine the proportion of HydF_EFG competent to activate HydA_EFG, a constant amount of HydF_EFG (0.01 nmol) was titrated with increasing amounts of HydA_EFG (0.07, 0.14, 0.35, 0.69, 1.38 and 3.46 nmol). Assays were prepared in triplicate as described above, and the observed rates of H2 evolution were converted to an activated fraction of HydA_EFG present in assays.

3. Results and discussion

3.1. Purification of the recombinant 6x-histidine tagged Hyd proteins: HydF_EFG is capable of conferring hydrogenase activity to HydA_EFG

The 6x-histidine tagged proteins eluted from the Ni2+ affinity column between 100 mM and 250 mM imidazole. In cases where the three Hyd accessory proteins were co-expressed, co-purification of non-His-tagged Hyd proteins was observed at low to intermediate imidazole concentrations (60–100 mM). For example, HydF co-eluted with HydE-His6 and HydG-His6, and HydE co-eluted with HydF-His6. At higher imidazole concentrations, however, pure 6x-histidine tagged protein was obtained.

Incubation of each of the Hyd maturation proteins (HydE, HydF, or HydG), purified from cellular extracts containing all three of these Hyd proteins, with inactive purified HydA_EFG revealed that neither HydE nor HydG alone has the ability to activate HydA_EFG. Purified HydF_EFG, however, was competent to activate HydA_EFG. The ability to activate HydA_EFG increased upon HydF_EFG purification, pointing to HydF_EFG as the sole species responsible for activation (Fig. 1A). In contrast, HydF_EFG was not competent to activate HydA_EFG, demonstrating an essential role for HydE and HydG in forming the activation-competent form of HydF. These results, which demonstrate the ability of purified HydF_EFG to activate HydA_EFG in the absence of any other proteins or small molecules, support our hypothesis that HydF serves as a scaffold for assembly of a cluster that is subsequently transferred to HydA_EFG, thus converting it to an active holoenzyme. The observation that HydE and HydG must be co-expressed with HydF, but are not required in the HydA_EFG activation assays, supports the hypothesis that although HydE and HydG serve to assemble a cluster precursor on HydF, they are not required for the subsequent transfer of the precursor to HydA. Furthermore, although HydF purified in these studies exhibits GTPase activity (data not shown), the presence of GTP does not affect the amount of activated HydA_EFG produced by HydF_EFG (data not shown), arguing against a direct role for GTP hydrolysis in cluster transfer to HydA_EFG.

The involvement of HydF as a specific scaffold protein in H-cluster biosynthesis is analogous to other complex cluster assembly pathways, in particular that of nitrogenase, where the FeMo cofactor is synthesized on a NifEN scaffold protein prior to being transferred to apo-nitrogenase to accomplish activation [11, 12]. In addition, the Isc and Suf systems for iron sulfur cluster assembly utilize specific scaffold proteins for the delivery of metal clusters to target apo-proteins [13, 14]. This report adds to what may be a common theme of complex metal cluster assembly occurring on a surrogate host until transfer occurs to form a holoenzyme.
3.2. Initial characterization of HydF

Purified HydF contains iron, however the stoichiometry depends on whether it is expressed in the absence (0.9 ± 0.2) or presence (1.6 ± 0.1) of a HydE/HydG background. UV–vis spectroscopic analysis of the as-isolated form of HydF shows a protein-centered band at 280 nm with additional features occurring at ~320, 420, 510 and 575 nm (Fig. 1B, solid black line). UV–vis analysis of HydFEG shows a protein-centered band at 280 nm with shoulders occurring at ~335 and 415 nm (Fig. 1B, solid red line). The spectroscopic features present in both HydFEG and HydFEG, which decrease in intensity upon addition of dithionite (Fig. 1B, dashed lines), are attributed to ligand to metal charge transfer bands characteristic of iron–sulfur clusters. This observed ability of HydF to bind iron–sulfur clusters is consistent with a previous report of the initial characterization of HydF from T. maritima [7]. The difference spectrum (HydFEG – HydFEG, Fig. 1B, inset) highlights the spectroscopic differences between the purified HydF proteins from the two different backgrounds. Notably, the difference spectrum exhibits features characteristic of [2Fe2S] clusters, such as those previously characterized in BioB [15]. These data indicate clear differences in cluster composition between HydFEG and HydFEG, suggesting that HydE and HydG affect the cluster composition of HydF. Further spectroscopic studies are underway to elucidate the differences between HydFEG and HydFEG.

3.3. Investigating the stoichiometry of HydAEGF activation by HydFEG

Maximal activation of HydAEGF by HydFEG was investigated by performing assays in which a constant amount of HydAEGF was titrated with increasing amounts of HydFEG (Fig. 2A). Based on the known activity of 304 μmol H₂ min⁻¹ mg⁻¹ for native [FeFe]-hydrogenase purified from Clostridium pasteurianum (see Section 2), these experiments indicate that approximately 15% of the heterologously expressed HydAEGF is capable of being activated in this process. The inability to accomplish full activation of HydAEGF may result from metal content heterogeneity and/or improper folding in purified HydAEGF, either of which might result from heterologous expression in the absence of the full complement of maturation proteins. In addition, a decreased percentage of HydAEGF activation is observed at higher HydFEG to HydAEGF ratios which is similar to effects observed for other systems in which scaffold proteins operate including the Isc and Nif systems [16,17].

To determine the fraction of HydFEG in our preparations capable of activating HydAEGF, a constant amount of HydFEG was titrated with increasing amounts of HydAEGF and the resulting amount of activated HydAEGF was determined. In this analysis, the lowest observed ratio of HydFEG to activated HydAEGF provides an estimate of the fraction of HydFEG molecules that are capable of activating HydAEGF. As can be seen in Fig. 2B, the ratio of HydFEG to activated HydAEGF decreases as the total HydAEGF present in the assay increases; the ratio approaches a limit of ~10 HydFEG per activated HydAEGF (Fig. 2B). The presence of excess HydAEGF presumably favors the transfer of the activating component from HydFEG to HydAEGF, and prevents the decrease in maturation observed at high ratios of HydFEG to HydAEGF as seen in Fig. 2A. These results reveal that ~10% of the HydFEG protein present in these preparations is competent to activate HydAEGF assuming a 1:1 activation stoichiometry. The observation that only ~10% of
HydFEG is competent for activation may suggest the existence of multiple cluster intermediates at differing stages of synthesis on HydFEG. This would be consistent with the role of HydF as a scaffold for the entire sequential assembly process. Unfortunately, given the partial occupancy of the activating component and the presumed heterogeneity of the system it is not possible to assign spectroscopic signatures of the activating component that would allow parallel characterization and quantification. Efforts are underway to increase the occupancy of the activating component bound to HydF to pave the way for detailed analysis.

3.4. Summary

The results presented herein provide the first functional analysis of [FeFe] hydrogenase maturation in a system using purified proteins, and identify HydF as acting in the terminal step of this process. Given the difference in iron content and spectroscopic features between HydF^EG and HydF^EG, the ability of purified HydF^EG alone to activate HydA^AEG, and the apparent functional similarity between HydF and other characterized scaffolds for iron–sulfur cluster assembly, we conclude that HydF operates as a scaffold or carrier protein in H-cluster assembly. Our proposed model for [FeFe] hydrogenase maturation involves HydE and HydG acting on HydF to assemble a cluster precursor, which is subsequently transferred to HydA to effect activation. This working model provides a strong foundation from which to base further experiments directed at elucidating the contributions of HydE, HydG, and other endogenous proteins on the formation of the remarkable H-cluster active site.

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Appendix A. Supplementary material


References


1. Materials and methods

1.1 Cloning the Hyd genes from C. acetobutylicum

In the case of the C. acetobutylicum Hyd proteins, only the 6x-histidine tagged constructs needed to be created, as the non-tagged proteins had already been cloned (12). The genes for HydE (GenBank accession no. CAC1631) and HydG (GenBank accession no. CAC1356) were engineered to contain BamHI and Sall, while the gene for HydF (GenBank accession no. CAC1651) was engineered to contain BamHI and HindIII sites. These restriction sites allow for the introduction of a N-terminal 6x-histidine tag on each of the proteins for purification when cloned into the MCSI on the Novagen Duet™ series of vectors. The genes for HydE, HydF, and HydG were cloned into the pETDuet, pRSFDuet, and pCDFDuet vectors, respectively. The following primers were utilized:

HydE-F:5'- GGATCCGATGGATAATATAATAAAGTTAA,
HydE-R:5'- GTCGACTTAACCAATAGATTCTTTGTAGCT,
HydF-F:5'-GGATCCGATGAATGAACTTAACTCAACAC,
HydF-R:5'- AAGCTTTTAGTTCCTACTCGATTGATTAATATTCTATCA,
HydG-F:5'-GGATCCGATGTATAATGTTAAATCTAAAG,
HydG-R:5'- CAGCTGTTAGAATCTAAAATCTCTTTGTCC.

The PCR products were gel purified and the correct size fragments isolated and ligated into the pGEM vector. Plasmid DNA from these colonies was isolated, digested with the appropriate restriction enzymes, and this fragment cloned into an expression vector (as indicated above), with sequencing information confirming the presence of the correct inserts.

1.2 Protein Expression in E.coli

Constructs encoding Hyd maturation proteins from C. acetobutylicum were transformed into E. coli- BL21 (DE3) (Stratagene) cells, respectively, for protein expression. Single colonies obtained from these transformations were grown overnight in LB media prior to inoculation of larger cultures. Two liter flasks containing 1 L of low salt (5 g/L) LB broth containing 5 g/L glucose and 50 mM potassium phosphate buffer pH 7.5 were inoculated with 5 ml/L inoculums and grown at 37 °C and 250 rpm shaking. These cultures were allowed to grow to an OD 600= 0.5 before being induced by the addition of IPTG to a final concentration of 1 mM. At the point of induction, 0.075 g/L ferrous ammonium sulfate (FAS) was also added. The cultures were grown an additional 2 hours, either at room temperature or 37°C at which time an additional aliquot of 0.075g/L FAS was added. The cultures were then transferred to a 10°C cold box and purged with N₂ overnight. Cells were harvested by centrifugation and the resulting cell pellets were stored at – 80°C until further use.

1.3 Protein Purification

All procedures were carried out under anaerobic conditions in a Coy chamber or in sealed bottles. Cell pellets were thawed and resuspended in a lysis buffer containing 20 mM sodium phosphate or 10 mM Hepes, pH 7.4, 0.5 M NaCl, 5% glycerol, 20 mM imidazole, 20 mM MgCl₂, 1 mM PMSF, 1% TritonX-100, 0.07 mg DNAse and RNAse per gram cell, ~ 0.6 mg lysozyme per gram cell. The lysis mixture was stirred for one hour, after which time the lysate was centrifuged in gas tight bottles at 38,000 x g for 30 minutes. The resulting extracts were loaded onto a 5 mL HisTrap™ HP Ni²⁺-affinity column (GE Healthcare) or a gravity flow column loaded with HisTrap™ HP Ni²⁺-affinity resin. In the case of utilizing the pre-packed column, the resin was pre-equilibrated with 20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl, 5% glycerol, 10 mM imidazole (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 20% to 50% to 100% buffer B (20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl, 5% glycerol, 500 mM imidazole), with each stage washing for 5 column volumes. Fractions of interest were pooled, dialyzed into 50 mM HEPES, pH 7.4, 0.5 M NaCl, and 5% glycerol, and concentrated using an Amicon concentrator fitted with a YM-10 membrane. For the gravity flow columns, a buffer with composition 20 mM Hepes, pH 7.4, 0.5 M NaCl, 20 mM imidazole (buffer C) was utilized for column equilibration and washing. Following column washing with 15-20 column volumes buffer C, protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 20% to 50% to 100% buffer D (20 mM Hepes buffer, pH 7.4, 0.5 M NaCl, 5% glycerol, 500 mM imidazole). Protein was flash frozen in liquid N₂ and stored at – 80 °C or in liquid N₂ until further use.