FEBS Letters 582 (2008) 2183–2187

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

Shawn E. McGlynn[a], Eric M. Shepard[a], Mark A. Winslow[a], Anatoli V. Naumov[a], Kaitlin S. Duschene[a], Matthew C. Posewitz[b,c], William E. Broderick[a], Joan B. Broderick[a,*], John W. Peters[a,*]

[a] *Department of Chemistry and Biochemistry, The Astrobiology Biogeocatalysis Research Center, Montana State University, Bozeman, MT 59717, United States*
[b] *Colorado School of Mines, Department of Chemistry and Geochemistry, Golden, CO 80401, United States*
[c] *National Renewable Energy Laboratory, Golden, CO 80401, United States*

Received 23 April 2008; accepted 24 April 2008

Available online 22 May 2008

Edited by Stuart Ferguson

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 *Clostridium acetobutylicum* were purified from heterologous expressions in *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.

Keywords: Hydrogenase; Metalloenzyme; Iron–sulfur cluster maturation; H-cluster; Scaffold

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 ligated 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 linked by a cysteinyl thiolate to a di-iron subcluster 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 *Chlamydomonas reinhardtii* defective in hydrogen production have revealed that products of the *hydEF* and *hydG* genes are required for the accumulation of active [FeFe] hydrogenase, and that *hydE*, *hydF*, and *hydG* are common to all organisms in which active [FeFe] hydrogenases are found [4]. Further studies have shown that the coexpression in *Escherichia coli* of HydE, HydF, and HydG from *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 *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 *E. coli* in which all three Hyd proteins from *C. acetobutylicum* are expressed simultaneously can activate heterologously expressed HydA [8]. The in vitro activation of HydA occurs only with extracts of *E. coli* in which all three Hyd maturation 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 *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 ($HydF^{EG}$) mediates [FeFe] hydrogenase activation, and HydF expressed in the absence of HydE and HydG ($HydF^{\Delta EG}$) is not able to effect activation. The activation of HydA expressed in a genetic background devoid of HydE, HydF, and HydG ($HydA^{\Delta EFG}$) by $HydF^{EG}$ occurs in a manner consistent with the previously described in vitro activation of $HydA^{\Delta EFG}$, but does not

*Corresponding authors. Fax: +1 406 994 7470.
E-mail addresses: jbroderick@chemistry.montana.edu (J.B. Broderick), john.peters@chemistry.montana.edu (J.W. Peters).

Abbreviations: $HydA^{\Delta EFG}$, HydA expressed in the absence of HydE, HydF, and HydG; $HydF^{EG}$, HydF co-expressed with HydE and HydG; $HydF^{\Delta EG}$, HydF expressed in the absence of HydE and HydG; AdoMet, S-adenosylmethionine

0014-5793/$34.00
doi:10.1016/j.febslet.2008.04.063

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 (His_6) 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 Ni^{2+}-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 6000i 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 ability of a given Hyd maturation protein to activate $HydA^{\Delta EFG}$ was tested by combining purified inactive $HydA^{\Delta EFG}$ with the respective purified Hyd maturase 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 $HydA^{\Delta EFG}$ (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 H_2 was monitored using gas chromatography as described previously [8].

2.4. Investigating the stoichiometry of $HydF^{EG}$ activating $HydA^{\Delta EFG}$

Experiments directed at determining the maximal activation of $HydA^{\Delta EFG}$ and the effects of increasing the amount of $HydF^{EG}$ were performed by adding increasing amounts of $HydF^{EG}$ (from 0.07 to 35 nmol) to a constant amount of $HydA^{\Delta EFG}$ (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 $HydA^{\Delta EFG}$ 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 H_2 min^{-1} mg^{-1} which was defined as 100% activity. In order to determine the proportion of $HydF^{EG}$ competent to activate $HydA^{\Delta EFG}$, a constant amount of $HydF^{EG}$ (0.01 nmol) was titrated with increasing amounts of $HydA^{\Delta EFG}$ (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 H_2 evolution were converted to an activated fraction of $HydA^{\Delta EFG}$ present in assays.

3. Results and discussion

3.1. Purification of the recombinant 6x-histidine tagged Hyd proteins; $HydF^{EG}$ is capable of conferring hydrogenase activity to $HydA^{\Delta EFG}$

The 6x-histidine tagged proteins eluted from the Ni^{2+} affinity column between 100 mM and 250 mM imidazole. In cases when 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-His_6 and HydG-His_6, and HydE co-eluted with HydF-His_6. 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 $HydA^{\Delta EFG}$ revealed that neither $HydE^{FG}$ nor $HydG^{EF}$ alone has the ability to activate $HydA^{\Delta EFG}$. Purified $HydF^{EG}$, however, was competent to activate $HydA^{\Delta EFG}$. The ability to activate $HydA^{\Delta EFG}$ increased upon $HydF^{EG}$ purification, pointing to $HydF^{EG}$ as the sole species responsible for activation (Fig. 1A). In contrast, $HydF^{\Delta EG}$ was not competent to activate $HydA^{\Delta EFG}$, demonstrating an essential role for HydE and HydG in forming the activation-competent form of HydF.

These results, which demonstrate the ability of purified $HydF^{EG}$ to activate $HydA^{\Delta EFG}$ 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 $HydA^{\Delta EFG}$, 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 $HydA^{\Delta EFG}$ 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 $HydA^{\Delta EFG}$ produced by $HydF^{EG}$ (data not shown), arguing against a direct role for GTP hydrolysis in cluster transfer to $HydA^{\Delta EFG}$.

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 [11,13,14]. 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 holo-enzyme.

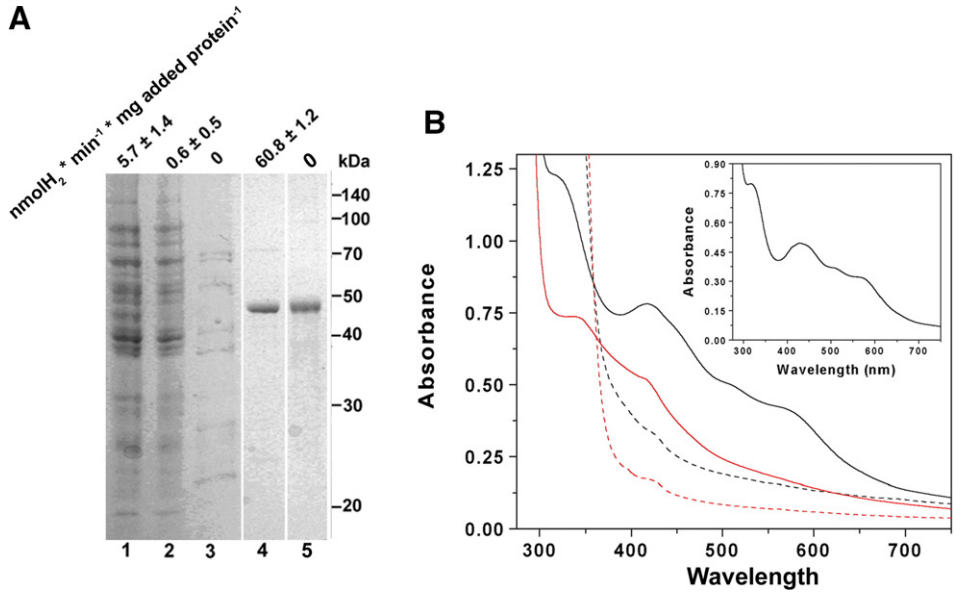

Fig. 1. (A) SDS/PAGE analysis (10% gel) showing IMAC purification of HydF co-expressed with HydE and HydG (lanes 1–4) and purified HydF expressed in the absence of HydE and HydG (lane 5). Lane contents left to right: 1 – clarified cell lysate, 2 – column flow through, 3 – 60 mM imidazole elution, 4 – 250 mM imidazole elution. Lane 5 – purified HydF expressed in the absence of HydE and HydG. Activation of $HydA^{\Delta EFG}$ was achieved by combining aliquots obtained during purification corresponding to the respective lanes with 4.4 μg $HydA^{\Delta EFG}$. Observed hydrogen evolution rates normalized to the amount of added protein are indicated above each lane. (B) UV–visible spectra of HydF expressed either singly (black trace, ~175 μM) or in the presence of HydE and HydG (red trace, ~170 μM). Dotted lines represent spectra obtained upon the addition of sodium dithionite. Inset: difference spectrum obtained by subtraction of iron-content-normalized spectra of the as-isolated proteins ($HydF^{\Delta EG}$ – $HydF^{EG}$) highlighting the differences between the two isolated forms of HydF.

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 $HydF^{\Delta EG}$ 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 $HydF^{EG}$ 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 $HydF^{\Delta EG}$ and $HydF^{EG}$, 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 ($HydF^{\Delta EG}$ – $HydF^{EG}$, 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 $HydF^{\Delta EG}$ and $HydF^{EG}$, suggesting that HydE and HydG affect the cluster composition of HydF. Further spectroscopic studies are underway to elucidate the differences between $HydF^{\Delta EG}$ and $HydF^{EG}$.

3.3. Investigating the stoichiometry of $HydA^{\Delta EFG}$ activation by $HydF^{EG}$

Maximal activation of $HydA^{\Delta EFG}$ by $HydF^{EG}$ was investigated by performing assays in which a constant amount of $HydA^{\Delta EFG}$ was titrated with increasing amounts of $HydF^{EG}$ (Fig. 2A). Based on the known activity of 304 μmol H_2 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 $HydA^{\Delta EFG}$ is capable of being activated in this process. The inability to accomplish full activation of $HydA^{\Delta EFG}$ may result from metal content heterogeneity and/or improper folding in purified $HydA^{\Delta EFG}$, either of which might result from heterologous expression in the absence of the full complement of maturation proteins. In addition, a decreased percentage of $HydA^{\Delta EFG}$ activation is observed at higher $HydF^{EG}$ to $HydA^{\Delta}EFG$ 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 $HydF^{EG}$ in our preparations capable of activating $HydA^{\Delta EFG}$, a constant amount of $HydF^{EG}$ was titrated with increasing amounts of $HydA^{\Delta EFG}$, and the resulting amount of activated $HydA^{\Delta EFG}$ was determined. In this analysis, the lowest observed ratio of $HydF^{EG}$ to activated $HydA^{\Delta EFG}$ provides an estimate of the fraction of $HydF^{EG}$ molecules that are capable of activating $HydA^{\Delta EFG}$. As can be seen in Fig. 2B, the ratio of $HydF^{EG}$ to activated $HydA^{\Delta EFG}$ decreases as the total $HydA^{\Delta EFG}$ present in the assay increases; the ratio approaches a limit of ~10 $HydF^{EG}$ per activated $HydA^{\Delta EFG}$ (Fig. 2B). The presence of excess $HydA^{\Delta EFG}$ presumably favors the transfer of the activating component from $HydF^{EG}$ to $HydA^{\Delta EFG}$, and prevents the decrease in maturation observed at high ratios of $HydF^{EG}$ to $HydA^{\Delta EFG}$ as seen in Fig. 2A. These results reveal that ~10% of the $HydF^{EG}$ protein present in these preparations is competent to activate $HydA^{\Delta EFG}$, assuming a 1:1 activation stoichiometry. The observation that only ~10% of

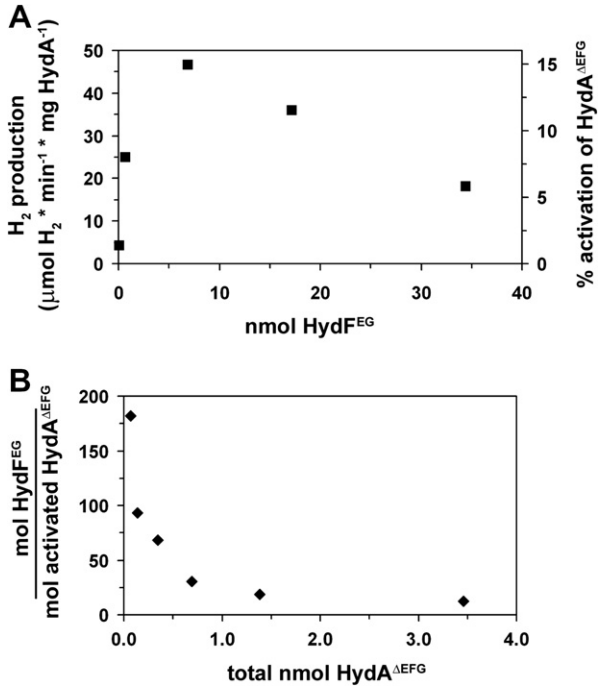

Fig. 2. (A) Rates of H_2 evolution and percent of $HydA^{\Delta EFG}$ activation as compared to the wild type enzyme activity are plotted as a function of the amount of $HydF^{EG}$ present in an assay mixture (0.07 to ~35 nmol) at constant $HydA^{\Delta EFG}$ (0.07 nmol). (B) Effect of increasing the amount of $HydA^{\Delta EFG}$ present in hydrogen production assays while holding the amount of $HydF^{EG}$ constant. Purified $HydF^{EG}$ (0.01 nmol) was titrated with increasing amounts of $HydA^{\Delta EFG}$ (0.07 to 3.46 nmol). The rates of hydrogen evolution were converted to a molar quantity of activated $HydA^{\Delta EFG}$ present in the reaction mixtures by comparing to the wild type isolated enzyme. The ratio of $HydF^{EG}$ to activated $HydA^{\Delta EFG}$ was plotted as a function of total $HydA^{\Delta EFG}$ present.

$HydF^{EG}$ is competent for activation may suggest the existence of multiple cluster intermediates at differing stages of synthesis on $HydF^{EG}$. 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 $HydF^{\Delta EG}$ and $HydF^{EG}$, the ability of purified $HydF^{EG}$ alone to activate $HydA^{\Delta EFG}$, 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.

Acknowledgements: We would like to thank Dr. Luis Rubio for helpful and insightful discussions. The work was supported by an AFOSR Multidisciplinary University Research Initiative Award (FA9550-05-01-0365) to J.W.P. and M.C.P. and the NASA Astrobiology Institute Funded Astrobiology Biogeocatalysis Research Center (NNA08C-N85A) award to J.B.B and J.W.P.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.04.063.

References

[1] Vignais, P.M. and Billoud, B. (2007) Occurrence, classification, and biological function of hydrogenases: an overview. Chem. Rev. 107, 4206–4272.
[2] Nicolet, Y., Lemon, B.J., Fontecilla-Camps, J.C. and Peters, J.W. (2000) A novel FeS cluster in Fe-only hydrogenases. Trend Biochem. Sci. 25, 138–143.
[3] Forzi, L. and Sawers, R.G. (2007) Maturation of [NiFe]-hydrogenases in *Escherichia coli*. Biometals 20, 565–578.
[4] Posewitz, M.C., King, P.W., Smolinski, S.L., Zhang, L., Seibert, M. and Ghirardi, M.L. (2004) Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. J. Biol. Chem. 279, 25711–25720.
[5] King, P.W., Posewitz, M.C., Ghirardi, M.L. and Seibert, M. (2006) Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. J. Bacteriol. 188, 2163–2172.
[6] Rubach, J.K., Brazzolotto, X., Gaillard, J. and Fontecave, M. (2005) Biochemical characterization of the HydE and HydG iron-only hydrogenase maturation enzymes from *Thermatoga maritima*. FEBS Lett. 579, 5055–5060.
[7] Brazzolotto, X., Rubach, J.K., Gaillard, J., Gambarelli, S., Atta, M. and Fontecave, M. (2006) The [Fe-Fe]-hydrogenase maturation protein HydF from *Thermotoga maritima* is a GTPase with an iron–sulfur cluster. J. Biol. Chem. 281, 769–774.
[8] McGlynn, S.E., Ruebush, S.S., Naumov, A., Nagy, L.E., Dubini, A., King, P.W., Broderick, J.B., Posewitz, M.C. and Peters, J.W. (2007) In vitro activation of [FeFe] hydrogenase: new insights into hydrogenase maturation. J. Biol. Inorg. Chem. 12, 443–447.
[9] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
[10] Fish, W.W. (1988) Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. Meth. Enzymol. 158, 357–364.
[11] Dos Santos, P.C., Dean, D.R., Hu, Y.L. and Ribbe, M.W. (2004) Formation and insertion of the nitrogenase iron–molybdenum cofactor. Chem. Rev. 104, 1159–1173.
[12] Rubio, L.M. and Ludden, P.W. (2005) Maturation of nitrogenase: a biochemical puzzle. J. Bacteriol. 187, 405–414.
[13] Frazzon, J. and Dean, D.R. (2003) Formation of iron–sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. Curr. Opin. Chem. Biol. 7, 166–173.
[14] Johnson, D.C., Dean, D.R., Smith, A.D. and Johnson, M.K. (2005) Structure, function, and formation of biological iron–sulfur clusters. Annu. Rev. Biochem. 74, 247–281.
[15] Ugulava, N.B., Sacanell, C.J. and Jarrett, J.T. (2001) Spectroscopic changes during a single turnover of biotin synthase: destruction of a [2Fe-2S] cluster accompanies sulfur insertion. Biochemistry 40, 8352–8358.

[16] Curatti, L., Hernandez, J.A., Igarashi, R.Y., Soboh, B., Zhao, D. and Rubio, L.M. (2007) In vitro synthesis of the iron molybdenum cofactor of nitrogenase from iron, sulfur, molybdenum, and homocitrate using purified proteins. Proc. Natl. Acad. Sci. USA 104, 17626–17631.

[17] Bonomi, F., Iametti, S., Ta, D. and Vickery, L.E. (2005) Multiple turnover transfer of [2Fe2S] clusters by the iron–sulfur cluster assembly scaffold proteins IscU and IscA. J. Biol. Chem. 280, 29513–29518.