Generation of Active [NiFe] Hydrogenase *in Vitro* from a Nickel-Free Precursor Form†

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ABSTRACT: The maturation process of [NiFe] hydrogenases includes formation of the nickel metallocenter, proteolytic processing of the large subunit, and assembly with the other hydrogenase subunit(s). An *in vitro* system for the maturation of the large subunit (HycE) of hydrogenase 3 of *Escherichia coli* leading to an active enzyme was established. The system is based on extracts of an *E. coli* mutant lacking the nickel-specific transport system (*nik*). HycE was present in these extracts in the C-terminally extended precursor form devoid of nickel. Addition of nickel led to nickel incorporation and proteolytic processing of HycE. Under anaerobic conditions, hydrogenase 3 activity was subsequently generated. The maximal rate of the processing reaction was reached at a nickel concentration of 400 μM. The accessory proteins known to be involved in the maturation of HycE *in vivo*, namely HypB, HypC, HypD, HypE, HypF, and the protease HycI, are required for the *in vitro* reaction, since processing of HycE did not occur in extracts of double mutants affected in the *nik* system and in one of the accessory genes. Processing of HycE and generation of hydrogenase 3 activity were achieved in extracts of the *nik*$^-$ Δ*hycI* mutant by addition of both nickel and purified HycI protease.

[NiFe] hydrogenases catalyze the production or consumption of hydrogen according to the reaction $2H^+ + 2e^- \leftrightarrow H_2$. The active site of the enzyme has been attributed to the nickel metallocenter [reviewed by Albracht (1994)], but its detailed structure is still a matter of investigation. X-ray crystallography of the *Desulfovibrio gigas* hydrogenase located the nickel center within the interior of the conserved large subunit and revealed the presence of a second metal (most probably iron) near the nickel atom. Nickel is liganded by four cysteines, two of which serve as bridging ligands to the second metal (Volbeda et al., 1995). In addition, the metallocenter appears to contain non-protein ligands which were detected by FTIR[1] spectroscopy and appear to be unique for hydrogenases (Bagley et al., 1995).

The biosynthesis of this metallocenter in the large subunit of hydrogenase requires the function of a conserved machinery of accessory proteins [reviewed by Maier and Böck (1996)]. In the case of hydrogenase 3 of *Escherichia coli*, seven genes (*hypABCDEF* and *hycI*) encoding some function in the maturation of the large subunit (HycE) have been identified (Lutz et al., 1991; Tomiyama et al., 1991; Rossmann et al., 1995).

Deletions in *hypA, hypB, hypC, hypD, hypE,* or *hypF* lead to a lack of nickel incorporation into HycE, indicating an involvement of the respective gene products in formation of the metallocenter (Jacobi et al., 1992; Maier et al., 1993, 1996). The HypB protein has been shown to exhibit GTPase activity (Maier et al., 1993) which is required for metallocenter formation (Maier et al., 1995). Its detailed biochemical function, however, and those of the other Hyp proteins are currently unknown.

The *hycI* gene encodes a processing protease (Rossmann et al., 1995) that removes a 32-amino acid peptide from the C-terminus of the HycE precursor protein (Rossmann et al., 1994). This reaction occurs only when nickel (and probably the other components of the active site) has already been incorporated into the precursor (Rossmann et al., 1994). Since the cleavage site is only three amino acids away from a cysteine of the active site, the processing reaction has been proposed to result in a structural rearrangement leading to a stable coordination of the metallocenter inside the protein (Rossmann et al., 1994). This notion was supported by the fact that direct synthesis of HycE in its mature form (devoid of the C-terminal extension) leads to an inactive enzyme lacking the nickel metallocenter (Binder et al., 1996).

Finally, hydrogenase activity is only established when processed HycE is assembled with all gene products required for activity, namely HycB, HycC, HycD, HycF, and HycG (Böhm et al., 1990; Sauter et al., 1992).

To investigate this unique process of the formation of a metallocenter in more detail, an *in vitro* system was developed. Until now, *in vitro* processing of a hydrogenase large subunit has been described in two cases. Purified protease HycI has been used for processing of HycE which was already loaded with nickel (Rossmann et al., 1994, 1995). In the case of the hydrogenase of *Azotobacter vinelandii*, the large subunit, present in extracts of cells starved of nickel, can be processed in a nickel-dependent manner *in vitro* (Menon & Robson, 1994). However, no instance has been reported where hydrogenase activity is restored *in vitro*. In this communication, we report on the maturation of hydrogenase 3 of *E. coli in vitro* using crude

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FTIR, fourier transform infrared; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

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Table 1: *E. coli* Strains

strain	genotype	reference
MC4100	F⁻, *araD193*, Δ(*argF-lac*)-*U169*, *ptsF25*, *relA1*, *flbB5301*, *rpsL150*, *λ*⁻	Casadaban and Cohen (1979)
HYD723	MC4100, *nikA*::Mud*I*	Wu and Mandrand-Berthelot (1986)
SMP101	MC4100, *hypA* (ATG → TAA)	Jacobi et al. (1992)
DHP-B	MC4100, Δ*hypB*	Jacobi et al. (1992)
DHP-C	MC4100, Δ*hypC*	Jacobi et al. (1992)
DHP-D	MC4100, Δ*hyp*D	Jacobi et al. (1992)
DHP-E	MC4100, Δ*hypE*	Jacobi et al. (1992)
DHP-F	MC4100, Δ*hypF*	Maier et al. (1996)
HD705	MC4100, Δ*hycE*	Sauter et al. (1992)
HD709	MC4100, Δ*hycI*	Binder et al. (1996)
NPA100	SMP101, *nikA*::Mud*I*	this work
NPB200	DHP-B, *nikA*::Mud*I*	this work
NPC300	DHP-C, *nikA*::Mud*I*	this work
NPD400	DHP-D, *nikA*::Mud*I*	this work
NPE500	DHP-E, *nikA*::Mud*I*	this work
NPF600	DHP-F, *nikA*::Mud*I*	this work
NCE500	HD705, *nikA*::Mud*I*	this work
NCI900	HD709, *nikA*::Mud*I*	this work

extracts that contain the nickel-free precursor form of HycE. Addition of nickel allows not only nickel incorporation and processing of HycE but also development of hydrogenase activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. All strains employed are derivatives of *E. coli* strain MC4100 (Table 1). Strain HYD723 carries a Mud*I* (Ap, *lac*⁺) insertion in the *nik* operon that encodes the nickel-specific transport system (Navarro et al., 1993). This mutation was transferred to strains with mutations in one of the accessory genes for HycE maturation by transduction using phage P1*kc* (Miller, 1992) and selection for ampicillin resistance. Transductants were tested for their *lac*⁺ phenotype and its repression in the presence of 500 μM $NiCl_2$ (Wu & Mandrand-Berthelot, 1986). *nik*⁻ mutants were grown anaerobically in TGYEP medium (Begg et al., 1977) containing 30 mM sodium formate and 50 μM EDTA. The cells were harvested at an optical density at 600 nm of 1.0 and washed with 50 mM Tris/HCl (pH 7.4).

In Vitro Processing Reaction. Cells were resuspended in 10 mM Tris/HCl (pH 7.4), and PMSF was added to a final concentration of 20 μg/mL. Extracts were prepared by passage through a French pressure cell at 118 MPa. They were centrifuged at 30000*g*, aliquoted, frozen in liquid nitrogen, and stored at −70 °C until use. No apparent decrease of the activity of such extracts for *in vitro* processing was observed over 3 months.

In standard reactions, the extracts (about 17.5 mg of protein/mL) were thawed, $NiCl_2$ was added to a final concentration of 400 μM, and the samples were incubated at 30 °C. At the indicated intervals, samples were withdrawn and mixed with SDS sample buffer (Laemmli, 1970) to stop further reaction. The samples were then subjected to SDS/polyacrylamide gel electrophoresis (10%), blotted onto nitrocellulose filters, and analyzed for reactions with anti-HycE antibodies (Sauter et al., 1992). The chemoluminograms obtained by detection with the ECL system (Amersham Buchler, Braunschweig) were further analyzed by laser densitometry, and the relative amount of HycE in the processed form was determined.

For analysis of nickel incorporation, processing was performed with an extract after 100000*g* centrifugation by addition of 160 μM nonradioactive $NiCl_2$ and 40 μM $^{63}NiCl_2$ (926 mCi/mmol). After 1 h of incubation at 30 °C, the extract was passed over a PD10 desalting column (Pharmacia Biotech, Freiburg) to remove unincorporated nickel. The eluate was further analyzed for cochromatography of HycE and radioactivity on a MonoQ column as previously described (Maier et al., 1993).

In Vitro Activation of Hydrogenase 3. In order to detect the formation of enzymatically active hydrogenase, extracts were prepared as indicated for *in vitro* processing reactions with the exception that the buffer contained 50 mM Tris/HCl (pH 7.4) and 1 mM DTT. The extracts were brought under a nitrogen atmosphere immediately after centrifugation at 30000*g*. Nickel was added to a final concentration of 400 μM, and the samples were incubated at 30 °C. At the given intervals, aliquots were withdrawn and analyzed for benzylviologen-dependent hydrogenase activity in anaerobic cuvettes according to Ballantine and Boxer (1985). When iron was added to the reaction mixture, a fresh $FeSO_4$ stock solution was prepared, degassed, and stored under nitrogen until use.

RESULTS

In Vitro Processing of HycE. To achieve the *in vitro* maturation of hydrogenase 3 of *E. coli*, the experiments were focused at first on the *in vitro* formation of processed large subunit (HycE) carrying the nickel metallocenter. Since the processing of HycE depends on accessory proteins, it was necessary to use crude extracts that contained these factors. In addition, HycE should be present as a substrate for nickel incorporation in the precursor form devoid of nickel. This situation should exist in extracts from cells lacking the nickel-specific transport system (*nik*). The *nikABCDE* operon encodes components of an ABC-type transporter (Wu et al., 1989; Navarro et al., 1993).

A *nik*⁻ mutant strain, HYD723, was grown anaerobically in rich medium containing 50 μM EDTA to eliminate unspecific nickel-uptake activity. Furthermore, formate was added to induce full expression of the *hycE* gene and of the accessory genes (Rossmann et al., 1991). As expected, HycE was present in the resulting crude extracts in the precursor form.

Upon addition of 400 μM nickel chloride and incubation at 30 °C, HycE was converted into the processed form (Figure 1A). When the amount of processed HycE was determined by laser densitometry (Figure 1B), it was found that within 20 min about 50% of the HycE was processed. After 2 h, the reaction stopped at about 70% conversion. A slow, "unspecific" processing of HycE took place in control experiments where no nickel had been added. This reaction is due to unspecific proteases of the extract, since it was independent of the presence of nickel and of the specific protease HycI (see below, Figure 4).

The processing reaction shown in Figure 1 was performed in 10 mM Tris/HCl (pH 7.4) under anaerobiosis, but assays carried out under aerobic conditions gave the same result. It was further examined whether addition of several reagents could enhance the reaction. Neither the precence of DTT (1 mM), NaCl (100 mM), ATP, or GTP (1 mM) nor the changing of the buffer [10 mM HEPES (pH 7.4)] or its molarity (10 to 100 mM Tris) had an obvious effect.

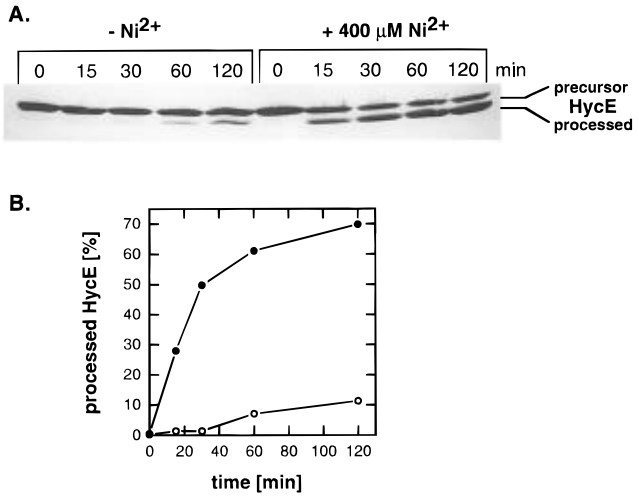

FIGURE 1: Kinetics of processing of HycE *in vitro*. (A) Chemoluminogram of samples from an extract of a nickel-uptake mutant (HYD723) incubated in the presence or absence of nickel. (B) Quantification of processed HycE by laser densitometry: samples after addition of 400 μM $NiCl_2$ (●) and samples without addition (○).

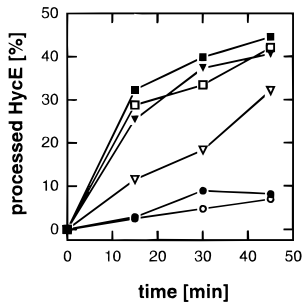

FIGURE 2: Dependence of HycE processing on the nickel concentration. Kinetics of *in vitro* processing reactions at different nickel concentrations, namely 0 μM (○), 20 μM (●), 100 μM (▽), 400 μM (▼), 800 μM (□), and 1.6 mM (■).

Moreover, addition of Fe^{2+} (400 μM) which is assumed to be a constituent of the metallocenter (Volbeda et al., 1995) had no significant influence on the reaction rate (data not shown).

In order to test whether the *in vitro* processing reaction is specific for nickel, other divalent cations were added to the extracts to a final concentration of 400 μM. Neither Fe^{2+}, Co^{2+}, Cu^{2+}, nor Zn^{2+} was able to substitute for Ni^{2+} (data not shown), indicating a high specificity of the maturation process for nickel. The K_m of the reaction for nickel could be roughly estimated to be 100 μM. Figure 2 shows the kinetics obtained with various nickel concentrations ranging from 0 to 1.6 mM. The reaction mixture was saturated with 400 μM nickel, whereas 100 μM nickel gave approximately a half-maximal rate.

To address the question of whether nickel is incorporated into HycE upon *in vitro* processing, a reaction was set up with the addition of ^{63}Ni. After 1 h of incubation, the extract was passed over a desalting column and analyzed for cochromatography of HycE and ^{63}Ni on a MonoQ column (Maier et al., 1993). Indeed, processed HycE was coeluted with a distinct peak of radioactivity indicating the association of ^{63}Ni with HycE (data not shown).

Formation of Hydrogenase Activity in Vitro. Next, it was investigated whether processing of the large subunit is accompanied by generation of hydrogenase activity. Therefore, the *in vitro* reactions were performed under anaerobic conditions, and samples were taken and analyzed for hydrogenase activity. Addition of 400 μM nickel chloride to extracts [prepared in buffer containing 50 mM Tris/HCl (pH 7.4) and 1 mM DTT] resulted in the generation of 0.7 unit of hydrogenase activity (Figure 3). This is about 12% of the activity present in extracts of the wild-type strain MC4100 (6.0 units) grown under identical conditions. Addition of Fe^{2+} had no influence on the reaction; if added alone, no activity was established (Figure 3), and if added together with Ni^{2+}, also no significant increase of the rate of appearance of activity was observed (data not shown).

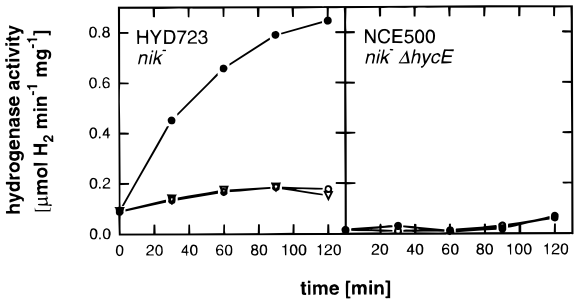

FIGURE 3: Kinetics of the generation of hydrogenase 3 activity *in vitro*. Extracts of the *nik*$^-$ strain HYD723 (left panel) and of a *nik*$^-$ Δ*hycE* mutant strain NCE500 (right panel) were incubated without addition (○), with 400 μM $NiCl_2$ (●), or with 400 μM $FeSO_4$ (▽) under anaerobic conditions. Samples were withdrawn and measured for benzylviologen-dependent hydrogenase activity (Ballantine & Boxer, 1985).

The activity formed was shown to be due to hydrogenase 3, because no activity was generated in the *nik*$^-$ Δ*hycE* double mutant (Figure 3). In principle, activation of the membrane-bound hydrogenases 1 and 2 could also take place, because the respective precursor proteins are present in the crude extract. Two reasons may account for the lack of significant activation of hydrogenases 1 and 2. First, the cells were grown under conditions under which hydrogenases 1 and 2 contribute only 5% to overall hydrogenase activity, and therefore, activation may be too low to be detected. Second, the activation of these enzymes is more complex, because it requires membrane integration. Without nickel addition, the extracts of the *nik*$^-$ mutant already displayed a hydrogenase activity of 0.1−0.2 unit. This background activity must be the result of incomplete nickel starvation of the cells and must be due to hydrogenase 3, because a *nik*$^-$ Δ*hycE* double mutant was devoid of it (Figure 3).

Requirement of in Vitro Processing for Accessory Components. For a comparison of the *in vitro* maturation of hydrogenase 3 with the *in vivo* reaction, it was important to analyze the requirement for the products of the *hyp* genes and the protease HycI. This was approached by using extracts of double mutants with lesions in the *nik* system and in one of the accessory genes (*hypA, hypB, hypC, hypD, hypE, hypF*, and *hycI*). Therefore, the *nikA*::Mu*d*I mutation of strain HYD723 was transduced into the respective *hyp* and *hycI* deletion mutants using phage P1. To verify the presence of both mutations, the resulting strains were tested for their expected phenotype. Gas production (due to hydrogenase 3 activity) in anaerobic media was restored in the double mutants only by suppression of the *nik*$^-$ mutation with 500 μM $NiCl_2$ plus introduction of a plasmid containing the respective accessory gene.

Figure 4 shows *in vitro* processing reactions obtained with extracts of these double mutants compared to the *nik*$^-$ mutant (here, a reaction under aerobic conditions is shown in contrast

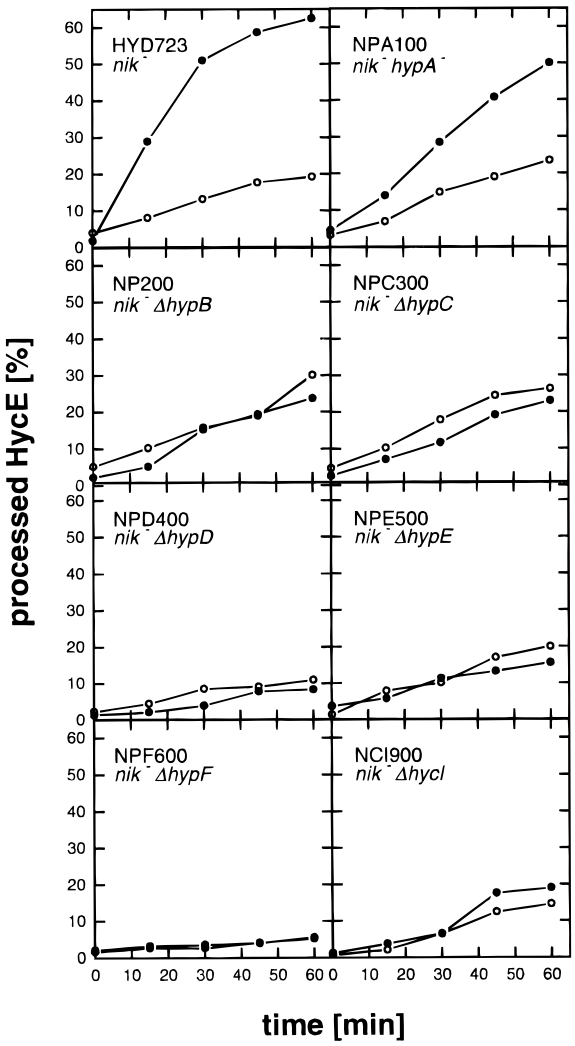

FIGURE 4: Effect of mutations in genes coding for accessory proteins on *in vitro* processing of HycE. Kinetics of HycE processing in different double mutants affected in the *nik* system and in one of the accessory genes (*hypA, hypB, hypC, hypD, hypE, hypF*, or *hycI*). Extracts were incubated with 400 μM $NiCl_2$ (●) or without addition (○) and subsequently analyzed for processing of HycE.

to the reaction in Figure 1). In the case of mutants affected in *hypB, hypC, hypD, hypE, hypF*, and *hycI*, nickel addition did not lead to specific processing of HycE. The extracts exhibited unspecific processing only. This reaction appeared to be significantly weaker in the *nik*$^-$ Δ*hypF* mutant; an observation that might indicate a decreased accessibility of the HycE precursor cleavage site. Extracts of a *nik*$^-$ *hypA*$^-$ mutant still showed a nickel-dependent processing reaction, but the reaction velocity was lower than that of the *nik*$^-$ mutant.

Functionality of Purified HycI Protease in Vitro. Since the accessory proteins are required for *in vitro* maturation, this system should provide an activity test for these proteins. As an example, the functionality of purified HycI was studied by means of extracts of the *nik*$^-$ Δ*hycI* mutant. In contrast to previous *in vitro* experiments with HycI (Rossmann et al., 1994, 1995), this assay requires nickel incorporation *in vitro* and allows for testing for formation of hydrogenase activity.

Purified HycI was indeed active in processing and formation of hydrogenase activity *in vitro* (Figure 5). The kinetics of processing and activation closely resemble those of a *nik*$^-$ mutant after addition of nickel (see Figures 1 and 3). It

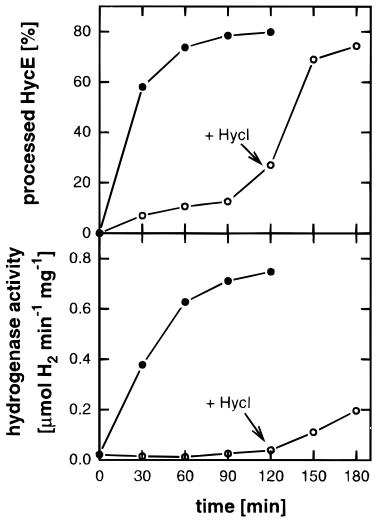

FIGURE 5: Functionality of purified HycI protease *in vitro*. Processing reactions (upper panel) and activation reactions (lower panel) were performed with extracts of a *nik*$^-$ Δ*hycI* double mutant after addition of 400 μM $NiCl_2$. Both reactions were carried out under anaerobic conditions in a buffer containing 50 mM Tris/HCl (pH 7.4) and 1 mM DTT. Purified HycI protease (0.5% of total protein) was added at the start point of the reaction (●) or after 2 h of incubation (○).

shows that purified HycI is as active as endogenous HycI in the case of the *nik*$^-$ mutant. An interesting result was observed when HycI was added to an assay already incubated with nickel for 2 h. The addition of the protease led to a rapid processing reaction, but only weak hydrogenase 3 activity appeared. Most likely, a factor (probably one of the Hyc components) required for activity (Sauter et al., 1992) was unstable upon prolonged incubation. The accessory proteins required for HycE processing apparently had retained their activity.

DISCUSSION

The investigation of *E. coli* mutants with defective hydrogenase 3 maturation had suggested the following order of events leading to an active hydrogenase: (i) formation of the nickel metallocenter within the large subunit (HycE), (ii) proteolytic processing near the C-terminus of HycE, and (iii) assembly of the hydrogenase enzyme complex (Jacobi et al., 1992; Maier et al., 1993; Rossmann et al., 1994). In the present communication, a system is introduced that allows the study of all of these steps in crude extracts *in vitro*. Most importantly, the *in vitro* maturation results in the generation of an active enzyme. A comparison of the kinetics of processing of HycE and activation of hydrogenase 3 shows that half-maximal processing of HycE is achieved after 20 min (Figure 1), whereas half-maximal enzyme activation requires about 40 min (Figure 3). These results and the experiments with HycI (Figure 5) confirm that HycE processing is a prerequisite, but not sufficient for formation of hydrogenase activity.

The characterization of the nickel-dependent *in vitro* processing step yielded results which are in good agreement with those obtained for *A. vinelandii* (Menon & Robson, 1994). Both systems are not affected by aerobiosis, and both require about 400 μM Ni^{2+} for an optimal reaction rate. In view of the nickel concentrations supporting optimal hydrogenase synthesis *in vivo* (<1 μM), this concentration is surprisingly high. However, it has to be considered that the concentration *in vivo* represents the extracellular concentra-

tion, and certainly, active transport leads to an accumulation within the cytoplasm. Moreover, nickel ions might be directly sequestered by the accessory proteins after transport *in vivo*; uncoupling of these processes *in vitro* may raise the requirement for nickel.

Another reason could be that nickel incorporation *in vitro* takes place independently of the accessory proteins, but this possibility was ruled out. Nickel-dependent processing was absent when extracts of Δ*hypB*, Δ*hypC*, Δ*hypD*, Δ*hypE*, Δ*hypF*, or Δ*hycI* mutant strains were used. Only extracts from a *hypA*$^-$ mutant allowed a slow but significant response to nickel addition. A possible explanation for this loose dependence on an intact *hypA* gene may reside in the presence of a homologous gene in the hydrogenase 2 operon (*hybF*) (Menon et al., 1994). Its gene product, HybF, may substitute for HypA in the reaction *in vitro*.

The dependence of *in vitro* processing on *hypB* might be unexpected, because *in vivo* a mutation in *hypB* can be partially suppressed by concentrations of 500 μM nickel in the growth medium (Waugh & Boxer, 1986). Recently, it has been shown that the effect of nickel addition on maturation is overestimated when hydrogenase activity is examined, because nickel addition allows only very weak processing of HycE (only 5−10% at 500 μM $NiCl_2$) (Maier et al., 1995). As a consequence, this reaction is expected to contribute to *in vitro* processing only very weakly at 400 μM, and indeed, no significant HypB-independent processing by nickel addition was observed. A requirement for HypB implies that GTP is consumed upon *in vitro* processing, because GTP hydrolysis by HypB has been found to be essential for metallocenter formation *in vivo* (Maier et al., 1995). However, addition of GTP to the extract did not stimulate *in vitro* processing, in contrast to the situation in *A. vinelandii* (Menon & Robson, 1994).

The use of extracts from double mutants affected in the *nik* system and in one of the accessory genes is promising for the establishment of activity tests for purified accessory proteins. In the case of the HycI protease, this was shown to be possible. However, from the experiments with the double mutants, it cannot be concluded that all the accessory proteins fulfill their function *in vitro* in our assays. It could be that part of the Hyp proteins had already acted on the HycE precursor *in vivo* before extract preparation and rendered it competent for nickel incorporation *in vitro*. For example, the second metal (designated Me) (Volbeda et al., 1995) or (and) the unusual ligands (designated L) of the active site (Bagley et al., 1995) may have already been incorporated into the HycE precursor of the *nik*$^-$ mutant *in vivo*.

In summary, the reported results combined with previous observations have identified at least four different intermediates of the HycE precursor with different susceptibility to *in vitro* and *in vivo* processing. (A) HycE precursor accumulated in mutants affected in *hypA*, *hypC*, *hypD*, *hypE*, or *hypF* is incompetent for processing. (B) HycE precursor present in a Δ*hypB* mutant is amenable to partial processing *in vivo* by addition of 500 μM nickel, but not *in vitro*. (C) HycE precursor from a *nik*$^-$ mutant shows processing *in vitro* and *in vivo* with 500 μM nickel added. (D) HycE precursor identified in a Δ*hycI* mutant is already loaded with nickel *in vivo* and can be processed *in vitro* by purifed HycI. An order of the formation of HycE intermediates A, B, and C cannot be postulated yet. Moreover, it is unclear whether HycE precursor forms with a partially assembled metallocenter (e.g. pre-HycE[L] or pre-HycE[Me]) exist or whether metallocenter formation is a highly coordinated process. Once the metallocenter [NiMeL] is formed in the HycE precursor (intermetiate D), processing by HycI and assembly with the other Hyc subunits takes place.

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REFERENCES

Albracht, S. P. J. (1994) *Biochim. Biophys. Acta 1188*, 167−204.

Bagley, K. A., Duin, E. C., Roseboom, W., Albracht, S. P. J., & Woodruff, W. H. (1995) *Biochemistry 34*, 5527−5535.

Ballantine, S. P., & Boxer, D. H. (1985) *J. Bacteriol. 163*, 454−459.

Begg, Y. A., Whyte, J. N., & Haddock, B. A. (1977) *FEMS Microbiol. Lett. 2*, 47−50.

Binder, U., Maier, T., & Böck, A. (1996) *Arch. Microbiol. 165*, 69−72.

Böhm, R., Sauter, M., & Böck, A. (1990) *Mol. Microbiol. 4*, 231−243.

Casadaban, M. J., & Cohen, S. N. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4530−4533.

Jacobi, A., Rossmann, R., & Böck, A. (1992) *Arch. Microbiol. 158*, 444−451.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Lutz, S., Jacobi, A., Schlensog, V., Böhm, R., Sawers, G., & Böck, A. (1991) *Mol. Microbiol. 5*, 123−135.

Maier, T., & Böck, A. (1996) in *Advances in Inorganic Biochemistry: Mechanisms of Metallocenter Assembly* (Hausinger, R. P., Eichhorn, G. L., & Marzilli, L. G., Eds.) pp 173−192, VCH Publishers Inc., New York.

Maier, T., Jacobi, A., Sauter, M., & Böck, A. (1993) *J. Bacteriol. 175*, 630−635.

Maier, T., Lottspeich, F., & Böck, A. (1995) *Eur. J. Biochem. 230*, 133−138.

Maier, T., Binder, U., & Böck, A. (1996) *Arch. Microbiol. 165*, 333−341.

Menon, A. L., & Robson, R. L. (1994) *J. Bacteriol. 176*, 291−295.

Menon, N. K., Chatelus, C. Y., DerVartanian, M., Wendt, J. C., Shanmugam, K. T., Peck, H. D., & Przybyla, A. E. (1994) *J. Bacteriol. 176*, 4416−4423.

Miller, J. H. (1992) *A short course in bacterial genetics*, Cold Spring Harbor Laboratory Press, Plainview, NY.

Navarro, C., Wu, L.-F., & Mandrand-Berthelot, M.-A. (1993) *Mol. Microbiol. 9*, 1181−1191.

Rossmann, R., Sawers, G., & Böck, A. (1991) *Mol. Microbiol. 5*, 2807−2814.

Rossmann, R., Sauter, M., Lottspeich, F., & Böck, A. (1994) *Eur. J. Biochem. 220*, 377−384.

Rossmann, R., Maier, T., Lottspeich, F., & Böck, A. (1995) *Eur. J. Biochem. 227*, 545−550.

Sauter, M., Böhm, R., & Böck, A. (1992) *Mol. Microbiol. 6*, 1523−1532.

Tomiyama, M., Shiotani, M., Sode, K., Tamiya, E., & Karube, I. (1991) in *Abstracts of 3rd international conference on the molecular biology of hydrogenases*, pp 24−25, Troia, Portugal.

Volbeda, A., Charon, M.-H., Piras, C., Hatchikian, E. C., Frey, M., & Fontecilla-Camps, J. C. (1995) *Nature 373*, 580−587.

Waugh, R., & Boxer, D. H. (1986) *Biochimie 68*, 157−166.

Wu, L.-F., & Mandrand-Berthelot, M.-A. (1986) *Biochimie 68*, 167−179.

Wu, L.-F., Mandrand-Berthelot, M.-A., Waugh, R., Edmonds, C. J., Holt, S. E., & Boxer, D. H. (1989) *Mol. Microbiol. 3*, 1709−1718.

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