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In Vivo and In Vitro Nickel-Dependent Processing of the [NiFe] Hydrogenase in *Azotobacter vinelandii*

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H_2 oxidation in *Azotobacter vinelandii* is catalyzed by a membrane-bound, αβ dimeric [NiFe] hydrogenase. Maturation of the enzyme involves cleavage of a putative N-terminal signal sequence in the β subunit and removal of 15 amino acids from the C terminus of the α subunit. Cells limited for nickel exhibited low hydrogenase activities and contained an apparently large form of the α subunit. Addition of nickel to such cells increased hydrogenase activities fivefold over 2 h. The increase in the first hour did not require transcription and translation and correlated with processing of the large form of the α subunit (pre-α) to the small form (α) resembling the α subunit from the purified enzyme. In vivo, pre-α appeared soluble whereas the majority of α was membrane bound. Processing of pre-α to α was reproduced in vitro in membrane-depleted extracts of nickel-limited cells. Processing specifically required the addition of Ni^{2+}, whereas Co^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} were ineffective. However, Zn^{2+}, Co^{2+}, and Cu^{2+} inhibited nickel-dependent processing. Mg-ATP and Mg-GTP stimulated processing, whereas anaerobic conditions and/or the addition of dithiothreitol and sodium dithionite was unnecessary. Processing was not inhibited by the protease inhibitors phenylmethylsulfonyl fluoride, E64, and pepstatin.

The aerobic, dinitrogen-fixing bacterium *Azotobacter vinelandii* oxidizes dihydrogen (H_2) to protons and electrons, which pass through the electron transport chain to O_2. The reaction is catalyzed by a membrane-bound [NiFe] hydrogenase that purifies as a heterodimer of β and α subunits with apparent molecular masses of 31 and 67 kDa, respectively, and contains 0.68 mol of nickel and 6.6 mol of iron per mol of enzyme (31). The β and α subunits of hydrogenase are encoded by the *hoxK* and *hoxG* structural genes (22). Fourteen additional, tightly clustered, accessory genes lie adjacent to the structural genes (2, 3, 10, 20, 22). Disruption of some of these genes destroyed O_2-dependent H_2 oxidation activity and resulted in the appearance of a large form (*hoxM*, *hoxL*, and *hypE* mutants) or a mixture of a large and a small form of the α subunit (*hoxZ*, *hoxO*, *hoxQ*, and *hoxR* mutants) (9, 20). This suggested that the α subunit normally undergoes a posttranslational modification that is perturbed in the mutants. The purified, mature α subunit is 16 residues smaller than the predicted *hoxG* gene product, and since it lacks only the methionine at the N terminus, it was suggested that a C-terminal cleavage event accounts for the difference in size (11). The proposed cleavage site is 3 residues after the sequence -Cys-Leu-Ala-Cys- in the C terminus of the α subunit, which is highly conserved in other [NiFe] hydrogenases and likely to contribute ligands to nickel in the nickel-active site of the enzyme (28). The possibility that C-terminal processing is linked to nickel insertion and maturation of hydrogenase led us to study the influence of nickel availability on hydrogenase in *A. vinelandii*.

MATERIALS AND METHODS

Chemicals and glassware. Rifampin, chloramphenicol, and dithiothreitol (DTT) were obtained from Boehringer Mannheim, Indianapolis, Ind. Nitrilotriacetic acid (NTA) was purchased from J. T. Baker Co, Phillipsburg, N.J. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and sodium salts of ATP and GTP were obtained from Sigma Chemical Co., St. Louis, Mo. Emulgen 913 was purchased from Karlan Chemical Corp, Torrance, Calif. All chemicals used were reagent grade or better. Glassware was soaked in 0.2 M nitric acid for at least 24 h and rinsed in deionized water prior to use.

Media and growth conditions. *A. vinelandii* CA (1) was grown aerobically at 30°C in a rotary shaker at 250 rpm under N_2-fixing conditions in nickel-sufficient Burk's medium (26) containing sucrose (2% [wt/vol]), $FeSO_4 \cdot 7H_2O$ (18 μM), $CaCl_2 \cdot 2H_2O$ (500 μM), $MgCl_2 \cdot 6H_2O$ (785 μM), and $Na_2MoO_4 \cdot 2H_2O$ (3 μM), without any added nickel or other trace elements. Nickel-limited medium was prepared by addition of NTA (50 μM) to nickel-sufficient Burk's medium (27). Nickel-supplemented medium contained $NiCl_2 \cdot 6H_2O$ (25 μM) in addition to NTA. Inhibitors were added, when required, at the following concentrations: rifampin, 100 μg/ml; chloramphenicol, 100 μg/ml; and CCCP, 100 μM. Cell density was measured with a Klett-Summerson colorimeter fitted with a green filter and expressed as Klett units per milliliter. A culture of *A. vinelandii* with a density of 100 Klett units contains 0.13 mg of total cell protein per ml.

Hydrogenase uptake assays. Whole-cell H_2 oxidation activity coupled to O_2 reduction was determined amperometrically (34). Hydrogenase specific activity is expressed as nanomoles of H_2 consumed per minute per 10 Klett units.

Western immunoblot analysis. Denatured polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17) in 7% acrylamide gels. Western blotting and immunoanalyses were performed as described previously (20). Purified *A. vinelandii* [NiFe] hydrogenase was a gift from D. J. Arp. Rabbit antibody raised against native *A. vinelandii* hydrogenase (Antibodies Inc., Davis, Calif.) was used at a dilution of 1:1,000. The antibody did not cross-react significantly with the hydrogenase β subunit because it is only weakly antigenic.

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Cell fractionation. All steps were carried out at 4°C.

(i) In vivo studies. Cells from log-phase batch cultures (50 ml) were harvested by centrifugation, resuspended in 3 ml of dilution buffer [Tris HCl, pH 7.5, 20 mM; $Mg(CH_3COO)_2$, 2 mM], and sonicated, with a microtip probe (Heat Systems model W-385) at 50% maximum output, three times in eight 5-s bursts. The cell sonicates were centrifuged at 10,000 × *g* for 15 min. The resultant supernatants (crude extracts [CE]) were centrifuged at 100,000 × *g* in a Beckman L5-75B ultracentrifuge fitted with an SW50.1 rotor for 120 min. The high-speed supernatants comprise the soluble fraction, i.e., cytoplasm, periplasm, and possibly some peripheral membrane proteins. The crude membrane pellets were washed by resuspension and centrifugation, first in 3 ml of washing buffer I (Tris HCl [pH 7.5], 20 mM; NaCl, 1 M) and then in 3 ml of washing buffer II (Tris HCl [pH 7.5], 20 mM; Na_2EDTA, 1 mM). The washed membrane pellets were overlaid with 300 μl of washing buffer II and stored, along with the CE and high-speed supernatant fractions, at −20°C. Prior to loading on SDS-PAGE gels, resuspended membrane samples were diluted fivefold and solubilized with Emulgen 913 (0.5% [vol/vol]) for 20 min at room temperature.

(ii) In vitro studies. Cells from 1 liter of mid-log-phase (125 to 150 Klett units at 540 nm), nickel-limited cultures were harvested and washed with 10 mM Tris HCl (pH 7.5) (buffer A). The washed cells were resuspended in 10 ml of buffer A and broken by two passages through a French pressure cell at 18,000 lb/in^2. The cell lysate was centrifuged at 10,000 × *g* for 20 min. The resultant supernatant or CE was aliquoted and stored at −80°C. Membranes were removed from the CE by centrifugation at 100,000 × *g* for 3 h, as described above. The high-speed supernatant was also aliquoted and stored at −80°C.

In vitro processing assays. Processing of the α subunit in vitro was determined in cell extracts incubated at 30°C for 2 h. Assays contained 15 or 30 μl of CE (19 mg of protein per ml) or high-speed supernatant (9 mg of protein per ml), prepared from nickel-limited cultures, in a final volume of 17.5 or 35 μl, respectively. The following additions were made as specified (final concentrations): $MgCl_2 \cdot 6H_2O$, 7.85 mM; GTP, 1 mM; ATP, 5 mM; $NiCl_2 \cdot 6H_2O$, 0.4 mM; and DTT, 5 mM. For metal replacement or competition experiments, the following ultrapure metal salts were added to a final concentration of 0.4 mM: $CaCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 5H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot 4H_2O$. Protease inhibitors were used at the following concentrations: phenylmethylsulfonyl fluoride, 1 mM; E64, 0.1 mM; pepstatin A, 1 μg/ml; and 1,10-phenanthroline, 1 mM. Reactions were started by the addition of $NiCl_2$ unless otherwise stated.

RESULTS

Effects of nickel on hydrogenase in vivo. Burk's medium contains sufficient contaminating nickel that nickel supplementation is not required to maximize whole-cell hydrogenase activity. Addition of the chelating agent NTA (50 μM) to this medium did not affect growth of *A. vinelandii* but lowered hydrogenase activity by more than 80%. When nickel (25 μM) was added to NTA-containing cultures, a fivefold increase in hydrogenase activity was observed over 2 h. The ability of NTA to scavenge trace nickel in Burk's medium was exploited to study the influence of nickel availability on the amount, form, and localization of hydrogenase in *A. vinelandii*. Western immunoblot analysis of hydrogenase in nickel-sufficient cells revealed a single small form of the α subunit (Fig. 1, lane 1), which corresponded in apparent size to the α subunit in the purified enzyme. However, nickel-limited cells contained a large form of the α subunit (lane 9). When nickel was added to the nickel-limited culture, the large form of the α subunit disappeared within 60 min while the amount of the small form increased steadily over a 3-h period (lanes 2 to 8).

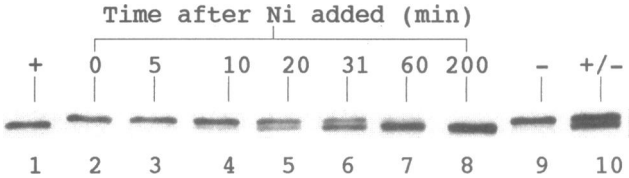

FIG. 1. Effects of NTA and nickel on the hydrogenase α subunit from *A. vinelandii* in vivo. Western blot analysis of the hydrogenase α subunit in whole-cell lysates from nickel-sufficient (lane 1), nickel-supplemented (lanes 2 to 8), and nickel-limited (lane 9) cultures. Lane 10 contains a 0.5:1 mixture of nickel-supplemented (lane 8) and nickel-limited (lane 9) whole-cell lysates.

The two forms of the α subunit occupy different locations in wild-type *A. vinelandii* cells (Fig. 2). The large form was found exclusively in the soluble fraction of nickel-limited cells (lane 6). The majority of the small form was membrane bound (lane 7), though some was observed in the soluble fraction of nickel-supplemented cells (lane 5).

Influence of inhibitors on nickel-induced effects on hydrogenase in vivo. When rifampin or chloramphenicol was added to nickel-limited cultures 2 min prior to nickel supplementation, hydrogenase activity increased over the first 70 min to the same extent as in an inhibitor-free control culture but ceased thereafter (Fig. 3). Hydrogenase activity continued to increase over 200 min in the nickel-supplemented control. The pattern of changes exhibited by the two forms of the α subunit during the initial 50 min was not affected by either inhibitor. This establishes that the large form is a precursor (pre-α) of the small form (α) and that the conversion is nickel dependent in vivo. Maximal hydrogenase activities in the inhibitor-treated cultures were attained approximately 20 min after the completion of processing. This shows that additional events are required for the enzyme to be active in whole-cell H_2 oxidation. The protonophore CCCP added 20 min prior to nickel-supplementation blocked both processing and accumulation of the α subunit (data not shown). No appreciable decrease was observed in the combined levels of pre-α and α in cells from any of the inhibitor-treated cultures during the experiments. This suggests that both pre-α and α are relatively stable.

Requirements for processing of pre-α in vitro. We were interested in determining whether processing of pre-α could be

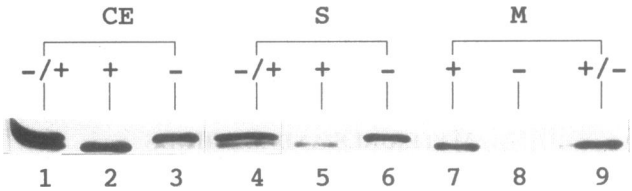

FIG. 2. Effect of nickel on the localization of the hydrogenase α-subunit forms in *A. vinelandii*. Western blot analysis of CE, soluble fractions (S), and membrane fractions (M) of nickel-limited (−) and nickel-sufficient (+) *A. vinelandii*. To demonstrate the two forms of the α subunit, 1:1 mixtures of nickel-limited and nickel-sufficient CE (lane 1), soluble fractions (lane 4), and membrane fractions (lane 9) were also included.

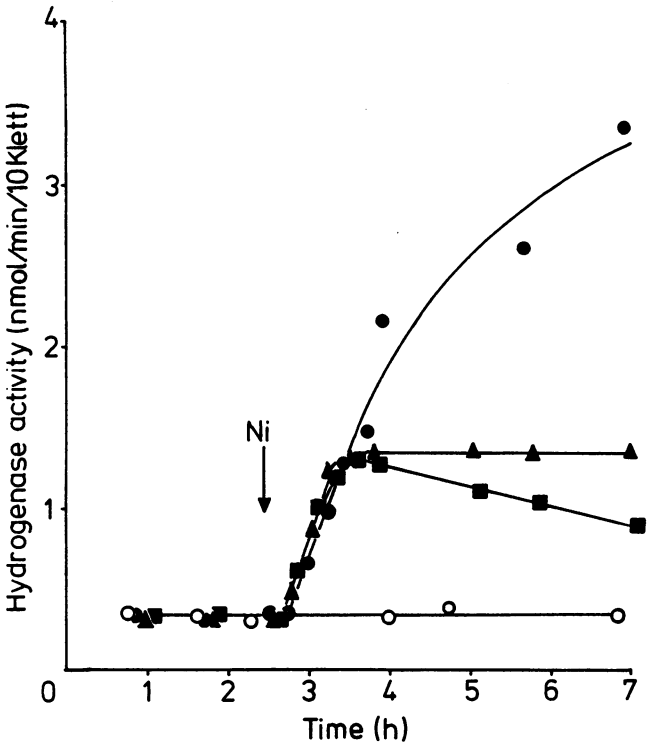

FIG. 3. Effects of inhibitors on in vivo hydrogenase activity in *A. vinelandii*. A nickel-limited overnight culture (50 ml) of *A. vinelandii* was diluted with fresh nickel-limited medium to 39 klett units/ml and divided equally (50 ml each) into four 250-ml flasks. Culture samples were withdrawn periodically and tested for hydrogenase activity. ○, nickel-limited control culture. Nickel (25 μM) was added to the remaining three cultures at the time indicated. ●, nickel-supplemented control culture; ▲, chloramphenicol added 2 min prior to nickel addition; ■, rifampin added 2 min prior to nickel addition.

reproduced in vitro. Cell extracts that contain pre-α were made from nickel-limited *A. vinelandii* cells. Pre-α in crude extracts underwent processing, but only after nickel was added. Mg-ATP and/or Mg-GTP was required to maximize processing. Nickel was most effective in the range of 200 to 800 μM, while levels greater than 1.6 mM were inhibitory. Approximately 50% of pre-α was processed within 45 min of nickel addition (Fig. 4); however, a small portion of pre-α remained unprocessed, even after 3 h. In several different experiments complete processing was never observed. Processing in CE was not

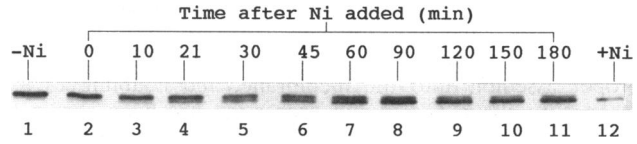

FIG. 4. In vitro processing of pre-α. Processing of the α subunit was followed by Western blot analysis of aliquots withdrawn periodically from an in vitro processing reaction. The assay mixture contained CE, prepared from nickel-limited *A. vinelandii* cultures, to which the following additions were made: Mg^{2+}, ATP, GTP, DTT, and Ni^{2+}. Lane 1, pre-α in CE of nickel-limited cells prior to any additions; lanes 2 to 11, time course of processing of pre-α, after the addition of nickel; lane 12, α subunit present in CE of nickel-supplemented cells, for comparison.

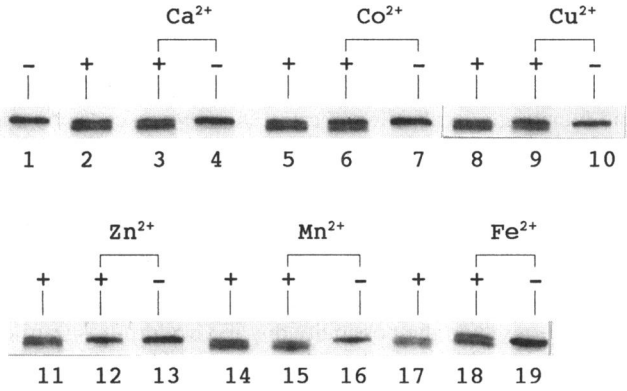

FIG. 5. Effect of metals on in vitro processing of pre-α. Western blot analysis of in vitro processing reactions with (+) or without (−) nickel added and in the presence of other divalent cations as shown above shown above the lanes. Reaction mixtures contained CE prepared from nickel-limited *A. vinelandii* cells, supplemented with Mg^{2+}, ATP, GTP, and DTT. Assays were started with the addition of nickel and/or the other divalent cations, and mixtures were incubated for 2 h at 30°C.

influenced by the presence of the thiol reductant DTT and occurred to the same extent under aerobic and anaerobic conditions. To determine whether membranes were required for in vitro processing, cell membranes were removed from crude extracts by high-speed centrifugation. Pre-α underwent processing to the same extent in the membrane-depleted extracts.

Effect of different metals and protease inhibitors on processing in vitro. We examined the ability of the divalent cations Zn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, and Mn^{2+} to substitute for or compete with Ni^{2+} in the processing reaction (Fig. 5). Ni^{2+} was the only cation tested which elicited processing. Zn^{2+} (400 μM) completely inhibited nickel-induced processing (lane 12). Co^{2+} and Cu^{2+} (400 μM) were also inhibitory, but to a lesser degree (lanes 6 and 9, respectively). Neither NaCl (0.4 mM) nor Na_2SO_4 (0.4 mM) affected nickel-dependent processing.

A set of protease inhibitors used to classify protease activities were tested for their ability to affect processing of pre-α in vitro. Phenylmethylsulfonyl fluoride (a serine protease inhibitor), E64 (a cysteine protease inhibitor), and pepstatin A (an aspartic peptidase inhibitor) had no effect on processing. However, the metal chelator 1,10-phenanthroline (a metalloprotease and metal-activated protease inhibitor) was inhibitory.

DISCUSSION

We proposed previously that C-terminal cleavage of the [NiFe] hydrogenase α subunit may be important in hydrogenase maturation, possibly linked to nickel insertion into the enzyme or its attachment to the membrane (11). The first idea is supported by the finding that nickel limitation in *A. vinelandii* results in the accumulation of an inactive, presumably nickel-deficient large form of the α subunit. Studies with protein synthesis and transcription inhibitors in vivo established that this form is the unprocessed precursor (pre-α) of the α subunit which is converted in the presence of nickel into the small or mature form (α) found in the active enzyme. Furthermore, processing of pre-α can be reproduced in cell extracts, where Ni^{2+} appears to be a highly specific requirement since equivalent levels of other divalent cations did not substitute. However, Zn^{2+} and, to a lesser extent, Co^{2+} and

Cu^{2+} inhibited nickel-induced processing. Possibly, these metals compete with nickel at one or more steps in the nickel-dependent processing pathway.

In vivo, there appears to be a correlation between processing and membrane localization because in wild-type cells pre-α is soluble while α is predominantly membrane bound. This raises the possibility that processing of pre-α occurs on the membrane. However, in vitro, processing occurs to the same extent in CE and extracts that have been depleted of membranes, suggesting that pre-α is processed in the cytoplasm prior to membrane attachment. Since membrane-depleted cell extracts combine both the cytoplasmic and periplasmic compartments, we cannot exclude the possibility that in vivo, pre-α is translocated across the inner membrane and processed in the periplasm. The pre-α C terminus may be involved in membrane translocation since C-terminal sequences are known to function as secretion signals in the export of proteins across both membranes of gram-negative bacteria (13). However, there appears to be no sequence identity between the C terminus of pre-α and these reported C-terminal signal sequences.

Processing of the α subunit in vivo was prevented by the protonophore CCCP. While the loss of the proton gradient might influence processing at any number of steps (e.g., nickel uptake), this observation may be correlated with the finding that Mg-ATP and Mg-GTP stimulate processing in vitro. At present we do not understand the basis for this requirement or know whether hydrolysis is required. However, we note that the *hypB* protein, required for nickel incorporation into hydrogenases in *Escherichia coli*, is a GTP-hydrolyzing protein (19). The equivalent *hypB* gene (ORF5 in reference 3) in the hydrogenase gene cluster of *A. vinelandii* contains a potential GTP-binding domain and a histidine-rich region which could bind nickel. We have recently purified the *A. vinelandii hypB* gene product in a single step on a nickel affinity column (35); therefore, the *hypB* protein may be a component of the pre-α processing machinery which interacts with both nickel and GTP.

Processing of pre-α is a surprisingly slow event. In vivo, the conversion is 50% complete within 20 to 30 min of the addition of nickel. The in vitro system reproduces these kinetics reasonably well (50% processing at 45 min) but does not go to completion even after 3 h. It is possible that factors necessary for processing are limiting in CE. Another intriguing possibility is that cell disruption converts a fraction of pre-α to a conformation that is refractory to processing.

We conclude that processing of pre-α involves a nickel-specific proteolytic event which is, in all likelihood, intimately associated with insertion of nickel into the polypeptide. Whether processing is a result of, or required for, nickel binding to ligands destined to form a part of the active site of the enzyme is yet to be determined. The cleavage could be autocatalytic and follow the binding of nickel to pre-α. Alternatively, proteolysis might be catalyzed by a specific protease which could contain nickel or be nickel dependent. Protease inhibitor studies on processing in CE appear to eliminate the involvement of serine, cysteine, and aspartate proteases. Partial inhibition of processing by 1,10-phenanthroline is indicative of a metalloprotease or metal-dependent protease but could also be a more general effect, e.g., chelation of nickel or other required divalent cations.

Maturation of other [NiFe] hydrogenases appears to involve events similar to those observed in *A. vinelandii*. An inactive, soluble or weakly membrane-bound form of the particulate [NiFe] hydrogenase has been described in nickel-limited, wild-type cells of *Alcaligenes eutrophus* (6). Large, and in some instances soluble, forms of the [NiFe] hydrogenase large subunit occur in hydrogenase structural and accessory gene mutant strains of *E. coli* (12, 18, 25, 30), *Bradyrhizobium japonicum* (7), and *A. eutrophus* (16) and when hydrogenase structural genes from *Desulfovibrio* species are expressed in *E. coli* (23, 24, 33). Many of these mutations also lead to unprocessed, membrane-bound forms of the hydrogenase small subunit (16, 25). In some instances, these phenotypes have been linked to defects in nickel assimilation (18, 19, 25). In addition, the large subunit of the periplasmic [NiFe] hydrogenase in *Desulfovibrio gigas* undergoes a C-terminal processing event similar to that observed in *A. vinelandii* (24). Also, processing of the *hycE*-encoded subunit of the formate hydrogen lyase in *E. coli* can occur in vitro when extracts from different hydrogenase mutants are mixed (12) and may involve a C-terminal cleavage.

On the basis of previously published work and the data presented here, we suggest the following general model for maturation of membrane-bound [NiFe] hydrogenases. In the absence of nickel, the hydrogenase subunits exist in nickel-waiting states, in which the α subunit is unprocessed and occurs in a stable conformation that is soluble and cannot enter the membrane, while the β subunit exists as a membrane-bound precursor that is probably arrested in export, since cleavage of the putative N-terminal signal sequence (a late event in export [29]) does not occur. The nickel-waiting state of the α subunit may be an incompletely folded form, possibly stabilized through association with a molecular chaperone that could be *hox* or *hyp* encoded. Chaperones regulate protein folding in a nucleotide-requiring and usually energy-dependent process (10), consistent with the observed ATP or GTP requirement for processing of pre-α. If the postulated chaperone were missing or nonfunctional (e.g., in *hox* or *hyp* mutants), abortive folding pathways could produce randomly folded polypeptides, some of which may insert in the membrane. This might explain why some *A. vinelandii* strains mutated in *hox* and *hyp* accessory genes contain pre-α in both the membrane and soluble cell fractions (9, 21).

Nickel incorporation into and/or C-terminal cleavage of pre-α may trigger correct folding into a form competent for nonabortive membrane insertion and possible export. Since no obvious signal exists for the export of the α subunit, it has been suggested that both subunits could be exported cooperatively (33). This would explain why only the β subunit contains a signal sequence and could ensure that neither subunit locks into the membrane until the metals Ni and Fe are correctly incorporated.

Experiments revealed some additional effects of nickel on the hydrogenase system in *A. vinelandii*. While there is a close correlation between the level of processed protein and in vivo activity, there is a lag between the completion of processing and the attainment of maximal hydrogenase activities. Thus, in addition to processing, further events are required for hydrogenase to be active in O_2-dependent H_2 oxidation, and these could involve the insertion of metal clusters into the enzyme, proper assembly into the membrane, and coupling to the respiratory chain. C-terminal processing has been shown to have a role in the assembly of active, membrane-bound protein complexes (4) and may have a similar function in hydrogenase maturation.

The in vivo inhibitor experiments showed that activation of preexisting enzyme by nickel does not require transcription or protein synthesis. This observation resembles the situation in *B. japonicum* mutants constitutive for hydrogenase formation, in which the hydrogenase formed in a nickel-deficient medium can also be activated in vivo by addition of nickel to the

medium (8). In addition, the inhibitor experiments in *A. vinelandii* showed that nickel appears to enhance de novo synthesis of hydrogenase because levels of hydrogenase protein were substantially greater in nickel-sufficient cells than in nickel-limited cells. Therefore, in this respect, the *A. vinelandii* system resembles those of *A. eutrophus* (5), *Azotobacter chroococcum* (27), *B. japonicum* SR (14, 32), and *Rhodospirillum rubrum* (15), in which nickel-dependent stimulation of hydrogenase activity requires protein synthesis. Whether transcription of hydrogenase genes in *A. vinelandii* has an absolute requirement for nickel, as in *B. japonicum* SR (32), cannot be judged from these experiments, since the growth medium was not completely depleted of nickel.

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