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Nickel Affects Expression of the Nickel-Containing Hydrogenase of *Alcaligenes latus*

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The effects of nickel on the expression of hydrogenase in the hydrogen-oxidizing bacterium *Alcaligenes latus* were studied. In the absence of added nickel, both hydrogenase activity, measured as O_2-dependent H_2 uptake, and hydrogenase protein, measured in a Western immunoblot, were very low compared with the levels in cells induced for hydrogenase in the presence of nickel. Hydrogenase activity and protein levels were dependent on the added nickel concentration and were saturated at 30 nM added Ni^{2+}. The amount of hydrogenase protein in a culture at a given nickel concentration was calculated from the H_2 uptake activity of the culture at that Ni^{2+} concentration. Between 0 and 30 nM added Ni^{2+}, the amount of hydrogenase protein (in nanomoles) was stoichiometric with the amount of added Ni^{2+}. Thus, all of the added Ni^{2+} could be accounted for in hydrogenase. Between 0 and 50 nM added Ni^{2+}, all the Ni present in the cultures was associated with the cells after 12 h; above 50 nM added Ni^{2+}, some Ni remained in the medium. No other divalent metal cations tested were able to substitute for Ni^{2+} in the formation of active hydrogenase. We suggest two possible mechanisms for the regulation of hydrogenase activity and protein levels by nickel.

Alcaligenes latus is a facultative hydrogen-oxidizing bacterium that expresses high levels of hydrogenase activity in autotrophic culture (5, 19). The *A. latus* hydrogenase, like several other hydrogenases (10), contains Ni and Fe and is a membrane-bound αβ dimer, with subunits of 67,000 and 34,000 daltons (19). The hydrogenase of *A. latus* is not constitutive; rather, expression is induced under autotrophic conditions in the presence of H_2 and is repressed by carbon substrates and high O_2 levels (5). Thus, regulation of hydrogenase expression in *A. latus* is similar to that in many other bacteria that express nickel hydrogenases.

The role of Ni in H_2 metabolism has become the focus of considerable attention. Nickel is essential for hydrogenase activity in many H_2-oxidizing bacteria (4, 13, 21, 23). In purified hydrogenases from *Desulfovibrio gigas* (3, 15), *Chromatium vinosum* (1), and *Methanobacterium thermoautotrophicum* (14), electron paramagnetic resonance signals associated with Ni undergo redox state-dependent changes, and a role for Ni in the catalytic cycle of these hydrogenases has been proposed (3, 24, 25). In addition to the putative catalytic role, Ni might be involved in the regulation of expression of nickel hydrogenases. Upon addition of Ni^{2+} plus chloramphenicol to various bacteria induced for hydrogenase in the absence of Ni^{2+}, active hydrogenase is not formed (7, 13, 16, 21, 28). This implies that Ni is not inserted into preformed hydrogenase apoenzyme; rather, protein synthesis is required for incorporation of Ni into hydrogenase. In *Bradyrhizobium japonicum*, both hydrogenase activity and hydrogenase protein levels are proportional to the levels of added nickel at low nickel concentrations, and it was proposed that nickel regulates hydrogenase expression in *B. japonicum* at the transcriptional level (21). However, no evidence for transcriptional control of the hydrogenase operon by Ni^{2+} in *Escherichia coli* was obtained when a *hyd*::*lacZ* fusion was used (29). Bacterial operons that are known to be regulated by metal ions include Hg resistance genes (22), the Fe assimilation pathways of *E. coli* (17), the Cu resistance genes of *Pseudomonas syringae* pv. *tomato* (D. A. Cooksey, personal communication), and the MoO_4^{2-}-regulated alternate nitrogenase system of *Azotobacter vinelandii* (11).

Although Pinkwart et al. (19) showed that the hydrogenase of *A. latus* contains Ni, nothing else is known about the characteristics of Ni metabolism in this organism. We have studied the effect of Ni on the expression of hydrogenase protein and activity in *A. latus* incubated under autotrophic conditions. *A. latus* hydrogenase activity was very low in the absence of added Ni^{2+}, but was expressed at normal levels upon addition of Ni^{2+}. In the absence of Ni^{2+}, little or no immunologically cross-reactive material accumulated in the cells. The expression of hydrogenase activity was saturated at very low Ni^{2+} levels. At subsaturating concentrations of added Ni^{2+}, the amount of hydrogenase produced appeared to be stoichiometric with the amount of Ni^{2+} added.

MATERIALS AND METHODS

Treatment of media to remove Ni. All glassware was washed for at least 12 h in 18 N H_2SO_4–8 N HNO_3 and rinsed three times with distilled, deionized H_2O. All plasticware was washed in 3 N HNO_3–2 N HCl, and rinsed in the same way as the glassware.

Both heterotrophic growth medium and autotrophic hydrogenase induction medium were based on the mineral salts medium of Palleroni and Palleroni (18) and modified as previously described (5). The autotrophic induction medium contained 3.40 mg of KH_2PO_4 ml^{-1}, 3.55 mg of Na_2HPO_4 ml^{-1}, 1 mg of NH_4Cl ml^{-1}, 30 μg of H_3BO_3 ml^{-1}, and 3 μg of Na_2MoO_4 ml^{-1}. Heterotrophic medium consisted of autotrophic induction medium plus 0.5% (wt/vol) glucose. Ni^{2+} was removed from the heterotrophic medium and the autotrophic induction medium by passage through a column (2.3 by 6 cm) of controlled pore glass (CPG)-8-hydroxyquinoline (Pierce Chemical Co., Rockford, Ill.). The pH of the media was maintained below 6 during the CPG-8-hydroxyquinoline treatment to maintain the integrity of the column material (6). The volumes of medium passed through the column were limited to 250 ml, after which the column was washed sequentially with 60 ml of distilled H_2O, 60 ml of 1.2 N HCl,

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and 60 ml of distilled H_2O. After passage through the column, the media were stored in new, high-density polyethylene containers. Before the media were used, the pH was adjusted to 7.0 by addition of isothermally distilled NH_4OH (26). Ultrapure trace element salts, stored as stock solutions in new polyethylene bottles, were added to the following final concentrations: $CaSO_4 \cdot 2H_2O$, 0.05 μg ml^{-1}; $MnSO_4 \cdot 4H_2O$, 0.07 μg ml^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 mg ml^{-1}; $Fe_2(SO_4)_3$, 0.06 μg ml^{-1}; $CuCl_2$, 0.008 μg ml^{-1}; $ZnCl_2$, 0.05 μg ml^{-1}; and $CoCl_2$, 0.11 μg ml^{-1}. All essential trace elements used in this study were either Puratronic grade (Johnson Matthey Chemicals, Ltd., Royston Hertfordshire, England) or Gold Label grade (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The trace elements were certified ≥99.995% pure by the manufacturers. All media were filter sterilized, and all additions were made with metal-free polypropylene pipette tips.

Growth of cells. *A. latus* H-1 (DSM 1123) was grown in heterotrophic medium as previously described (5). The medium contained no added Ni^{2+} and was purified of contaminating Ni^{2+} as described above. A 50-μl portion from a heterotrophic starter culture, grown in air at 25°C with shaking at 170 rpm, was used to inoculate 20 ml of Ni^{2+}-depleted heterotrophic medium in a 50-ml acid-washed Erlenmeyer flask. After this culture had grown to mid-log phase (optical density at 600 nm, 2.0 to 2.3), 50 μl was used to inoculate a second, identical flask. After growth of the culture, again to mid-log phase, 2 to 3 ml was added to 200 ml of Ni^{2+}-depleted heterotrophic medium in an acid-washed 1-liter flask. This culture was grown to mid-log phase, and the cells were harvested by centrifugation at 8,000 × *g* for 10 min in an acid-washed 250-ml polyethylene centrifuge bottle. The cells were washed twice with 200 ml of Ni^{2+}-depleted autotrophic medium and centrifuged as above. They were then resuspended in 20 ml of Ni^{2+}-depleted autotrophic medium. All resuspensions and transfers were performed with sterile polystyrene pipettes or sterile metal-free polypropylene pipette tips.

Induction of hydrogenase activity. All experiments were performed essentially as described previously (5) with acid-washed, 160-ml stoppered serum bottles. Resuspended heterotrophically grown *A. latus* cells (1 ml) were added to 15 ml of nickel-depleted autotrophic medium under an atmosphere of 80% H_2–10% O_2–10% CO_2. Incubations were performed at room temperature for 12 or 24 h, as indicated, with shaking at 170 rpm. Added Ni^{2+} was Puratronic grade $NiCl_2$. O_2-dependent H_2 uptake activity was measured amperometrically (27) at 30°C in air-saturated 50 mM potassium phosphate buffer (pH 7.0). The electrode was calibrated by using H_2-saturated 50 mM potassium phosphate buffer (pH 7.0).

Western blot. All Western immunoblots were performed as previously described (5). Low levels of background staining in the Western blots were considered to be nonspecific because they appeared in all samples at equal intensities regardless of the hydrogenase activities associated with the cell samples. In addition, as noted previously (5), the hydrogenase levels were determined from the staining of the large subunit, since the hydrogenase small subunit does not react well in a Western blot.

Measurement of Ni uptake. $^{63}NiCl_2$ was obtained from Amersham Corp., Arlington Heights, Ill. The concentration of Ni^{2+} in this solution was determined colorimetrically by using a solution of 1 M CH_3COOH–3 M NH_4OH–0.5 mM 4-(2-pyridylazo)-resorcinol and was found to be 33.7 μg ml^{-1}, substantially different from the value supplied by the manufacturer (9.3 μg ml^{-1}). All calculations were performed with the experimentally determined Ni^{2+} concentration. Hydrogenase activity was induced in the presence of increasing ^{63}Ni-labeled $NiCl_2$ concentrations for 12 h. After induction, the cells were collected by centrifugation at 10,000 × *g* for 10 min. A sample of the supernatant was retained for scintillation counting, and the cells were twice washed with 10 ml of 50 mM potassium phosphate buffer (pH 7.0) and then resuspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.0). Samples (0.5 ml) of resuspended cells and supernatants were added to 10 ml of Aquasol liquid scintillation cocktail (Du Pont, NEN Research Products, Boston, Mass.), and the ^{63}Ni content was determined by scintillation counting in a ^3H window for 2 min. Efficiencies of counting were determined for each sample type (cells and supernatant) by the internal-standard method.

Protein assay. Assays of the total protein content of cell suspensions were performed as described previously (5). For the time course experiments (see Fig. 1 and 7), 50 μl of the cells taken at each time point was digested by adding 5 μl of 10 N NaOH and heating the mixture at 60°C for 30 min. The protein concentration in the digests was measured by using a protein assay reagent (Bio-Rad technical bulletin 1051, 1977; Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard.

Reagents. Electrophoresis chemicals, the protein assay kit, and metal-free polypropylene pipette tips were from Bio-Rad. Acrylamide was obtained from Eastman Kodak Co., Rochester, N.Y. Nitrocellulose paper used in Western blots was from Micro Filtration Systems, Dublin, Calif. The goat anti-rabbit–alkaline phosphatase conjugate used in Western blots was from TAGO, Inc., Burlingame, Calif. All Puratronic grade salts were obtained from Johnson Matthey Chemicals through Morton Thiokol, Inc., Alfa Products, Danvers, Mass. All other chemicals were reagent grade and were obtained from Fisher Scientific Co., Pittsburgh, Pa.; Sigma Chemical Co., St. Louis, Mo.; or Mallinckrodt, Inc., St. Louis, Mo. Gases were obtained from Liquid Carbonic Corp., Chicago, Ill., and were at least 99.5% pure.

RESULTS

Removal of Ni from growth and induction media. To determine the effect of Ni on the expression of hydrogenase protein and activity in *A. latus*, it was necessary to rigorously remove Ni from all media and glassware. We used the chelation chromatography matrix CPG-8-hydroxyquinoline to strip heterotrophic growth and autotrophic induction media of all divalent metal cations and then added ultrapure trace elements to these media (see Materials and Methods). Heterotrophic growth of *A. latus* in nickel-depleted medium occurred at similar rates and resulted in similar final densities as those produced in nickel-sufficient medium, even after repeated transfers (data not shown). This indicates that there is little or no Ni requirement for heterotrophic growth of *A. latus*.

Effect of Ni on hydrogenase expression in *A. latus*. When *A. latus* was grown heterotrophically in Ni^{2+}-depleted medium and then transferred to Ni^{2+}-depleted autotrophic medium under hydrogenase-inducing conditions, only low levels of hydrogenase activity developed (Fig. 1). These low levels ranged from <1 to 11% of the maximal activity. The highest levels of background hydrogenase activity could have arisen from as little as 0.6 ppb (10 nM) Ni^{2+}. Western blot examination of the levels of hydrogenase protein in cells induced in the absence of added Ni^{2+} showed that no

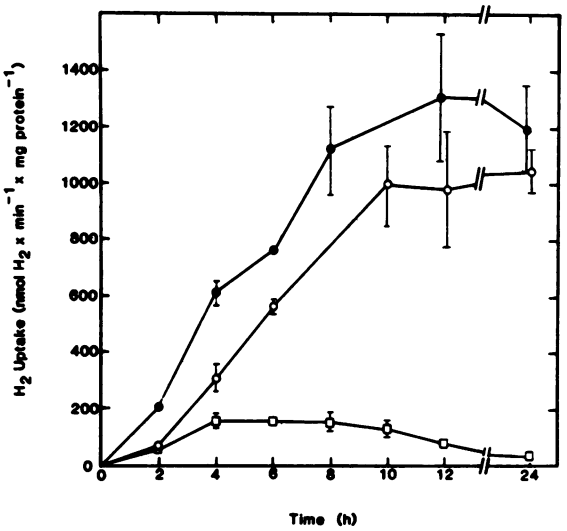

FIG. 1. Effect of Ni depletion on induction of hydrogenase activity in *A. latus*. Cells were grown and induced for hydrogenase activity as described in Materials and Methods. At the indicated time points, 0.3-ml samples were removed and the O_2-linked hydrogenase activities were determined amperometrically. The H_2 uptake values are the means (± standard deviations) of triplicate cultures. Symbols: ●, H_2 uptake activity in cells induced in autotrophic medium not treated to remove Ni^{2+}; □, H_2 uptake activity in cells induced in Ni^{2+}-depleted autotrophic medium; ○, H_2 uptake activity in cells induced in Ni^{2+}-depleted autotrophic medium plus 200 nM $NiCl_2$.

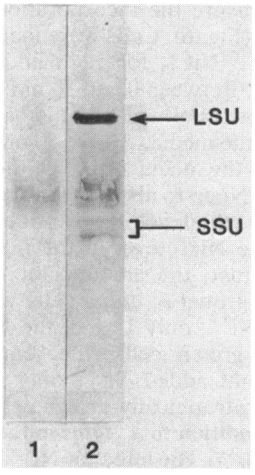

FIG. 2. Western blot of *A. latus* cells induced for hydrogenase activity in the presence and absence of Ni^{2+}. The cells were grown and induced for hydrogenase activity in the presence or absence of Ni^{2+} for 24 h as described in Materials and Methods. They were then collected by centrifugation and suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.0). Triton X-100-solubilized cell extracts (25 μg of cell protein) were electrophoresed, electroblotted onto nitrocellulose paper, and stained for hydrogenase protein as previously described (5). Lanes: 1, extract from cells induced in Ni^{2+}-depleted medium; 2, extract from cells induced in Ni-sufficient medium. Abbreviations: LSU, hydrogenase large subunit; SSU, hydrogenase small subunit.

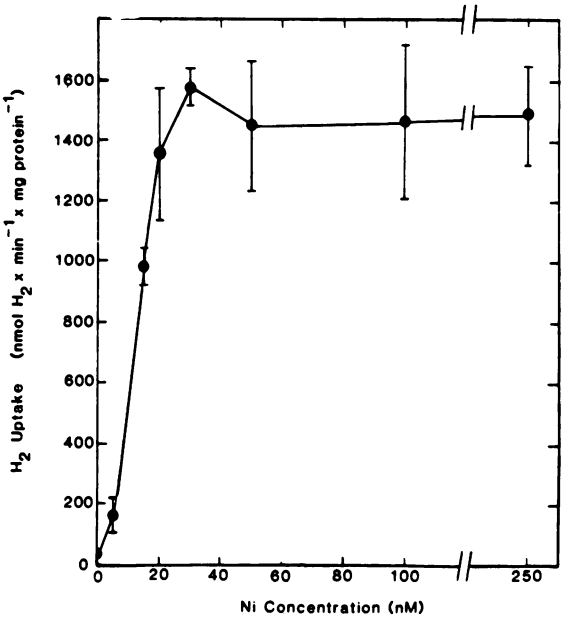

FIG. 3. Effect of medium Ni^{2+} concentration on hydrogenase expression in *A. latus*. Cells were induced for hydrogenase activity in the presence of the indicated amounts of $NiCl_2$. After 12 h, the cells were collected by centrifugation and suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.0). They were then assayed amperometrically for hydrogenase activity. Samples were assayed for total protein content as described previously (5). Total protein in the fully induced cultures ranged from 0.09 to 0.23 mg ml^{-1}. H_2 uptake values are the means (± standard deviations) of at least triplicate cultures.

immunologically detectable hydrogenase protein was present (Fig. 2). When cells were induced in the presence of 200 nM Ni^{2+}, high levels of hydrogenase protein were detected. In this experiment, hydrogenase activities in these cells induced in the absence and presence of added Ni^{2+} were 4 and 1,133 nmol of H_2 min^{-1} mg of cell $protein^{-1}$, respectively.

These results led us to determine the range of Ni^{2+} concentrations over which hydrogenase activity was expressed. Cells of *A. latus* were induced for hydrogenase activity in the presence of various concentrations of added Ni^{2+} (Fig. 3). Hydrogenase activity was very low when no added Ni^{2+} was present and rose with increasing Ni^{2+} concentration until it reached a maximum at 30 nM Ni^{2+}; it remained high up to 1 μM added Ni^{2+} and then dropped (data not shown). This decline was probably due to undefined Ni toxicity. A Western blot of cells induced for hydrogenase in the presence of various Ni^{2+} concentrations showed that hydrogenase protein levels correlated with activity (Fig. 4). Although the background hydrogenase activity varied, as described above, the levels of hydrogenase protein always correlated with the hydrogenase activity. This indicated a close relationship between hydrogenase activity and hydrogenase protein.

Correlation between hydrogenase protein levels and Ni^{2+} levels. An estimate was made of the amount of hydrogenase protein present in cultures by measuring the hydrogenase activity of the cells in each culture and knowing the specific activity of purified *A. latus* hydrogenase. We assumed (i) an in situ hydrogenase specific activity of 113 μmol of H_2 min^{-1} mg of $protein^{-1}$, equal to the specific activity reported by Pinkwart et al. for the purified enzyme (19); and (ii) a

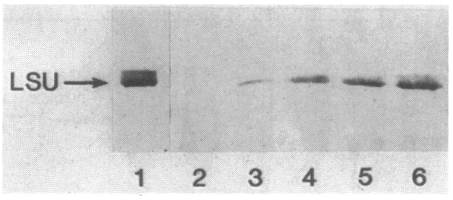

FIG. 4. Western blot of *A. latus* cells induced for hydrogenase activity in the presence of various Ni^{2+} concentrations. Cells of *A. latus* were induced for hydrogenase activity in media containing added $NiCl_2$ at the indicated concentrations for 24 h, as described in Materials and Methods. The cells were collected by centrifugation and suspended in 1 ml of potassium phosphate buffer (pH 7.0). Triton X-100-solubilized cell extracts (25 μg of cell protein) were electrophoresed, electroblotted onto nitrocellulose paper, and stained for hydrogenase protein as previously described (5). Lanes: 1, purified *A. latus* hydrogenase (0.75 μg); 2, 0 nM Ni^{2+}; 3, 10 nM Ni^{2+}; 4, 15 nM Ni^{2+}; 5, 20 nM Ni^{2+}; 6, 100 nM Ni^{2+}. Abbreviation: LSU, hydrogenase large subunit.

molecular weight of 101,000 for the hydrogenase (19). Using these values, we calculated the amount of hydrogenase protein in each culture and plotted this as a function of the amount of Ni^{2+} added to the culture (Fig. 5). The units of hydrogenase concentration and Ni^{2+} concentration were nanomoles per culture; this emphasized that the distribution of hydrogenase and Ni^{2+} throughout the culture was probably not uniform. Between 0 and 0.4 nmol of Ni^{2+} $culture^{-1}$, the amount of hydrogenase protein closely matched the amount of added Ni^{2+}. This suggests that most, if not all, of the added Ni^{2+} was taken up by the cells and incorporated into hydrogenase. At 0.4 nmol of Ni^{2+} $culture^{-1}$, the Ni^{2+} concentration was initially 25 nM in solution. Above 0.4 nmol of Ni^{2+} $culture^{-1}$, the hydrogenase activity did not increase, and thus the calculated amount of hydrogenase protein also remained constant. The slope of the line calculated by linear regression of the datum points between 0 and 0.4 nmol of Ni $culture^{-1}$ was 1.04. This indicates a Ni-to-hydrogenase stoichiometry of approximately 1.

Partitioning of Ni^{2+} between cells and medium. To further examine the correlation between the amount of hydrogenase

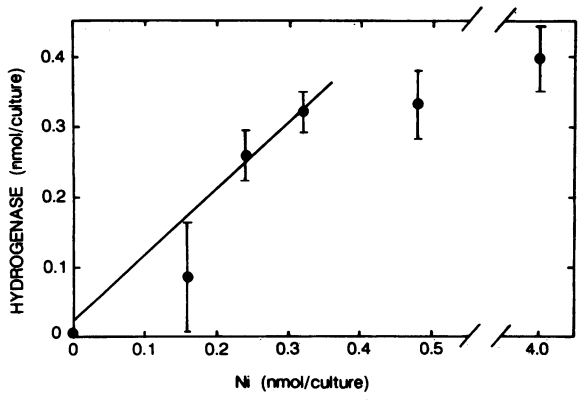

FIG. 5. Estimated hydrogenase concentration in *A. latus* plotted against Ni^{2+} concentration in culture. The total hydrogenase activity of each culture (in nanomoles of H_2 per minute) was divided by the specific activity of *A. latus* hydrogenase (113 μmol of H_2 min^{-1} mg of $protein^{-1}$ [19]) and the molecular mass of *A. latus* hydrogenase (101,000 daltons [19]) to give the number of nanomoles of hydrogenase in each culture. The line was obtained by linear regression analysis of the datum points between 0 and 0.4 nmol of Ni^{2+} $culture^{-1}$. Each point is the mean (± standard deviation) of at least triplicate cultures at each Ni^{2+} concentration.

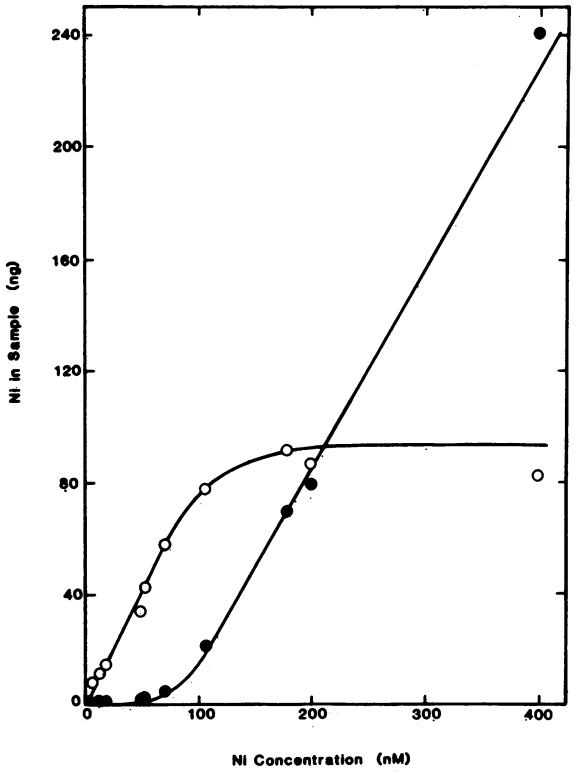

FIG. 6. Uptake of $^{63}NiCl_2$ by *A. latus* cells. Cells were induced for H_2 uptake activity for 12 h in Ni^{2+}-depleted media with the indicated added $^{63}NiCl_2$ concentrations. Ni uptake by cells was measured as described in Materials and Methods. All Ni uptake values are means of duplicate cultures. Symbols: ●, $^{63}Ni^{2+}$ in medium; ○, $^{63}Ni^{2+}$ in cells.

protein and the amount of added Ni^{2+}, we performed an experiment to measure the incorporation of added $^{63}NiCl_2$ into *A. latus* cells (Fig. 6). Cells were incubated with various concentrations of $^{63}NiCl_2$ for 12 h under hydrogenase-inducing conditions. Between 0 and 50 nM added Ni^{2+}, nearly all the Ni^{2+} was incorporated into the bacteria and virtually none appeared in the media. Above 50 nM added Ni^{2+}, Ni^{2+} began to appear in the media, although the cells accumulated larger amounts of Ni up to about 200 nM added Ni^{2+}. Above this concentration, the level of Ni in the cells remained the same, whereas the Ni^{2+} level in the medium continued to increase. In contrast, the amounts of Ni associated with heterotrophically grown *A. latus* cells were relatively low. At 50 nM added Ni^{2+}, only 14% of the Ni was found in the heterotrophically grown cells; the remainder was in the medium. At 200 nM added Ni^{2+}, only 10% of the Ni was found in the heterotrophically grown cells.

Effect of Ni^{2+} addition to *A. latus* induced for hydrogenase in the absence of Ni^{2+}. The effect of Ni^{2+} addition to *A. latus* induced for hydrogenase activity in the absence of Ni^{2+} was examined (Fig. 7). In the absence of Ni^{2+}, very low levels of hydrogenase activity were detected over the time course of the experiment. When Ni^{2+} was added to cultures after 6 h of induction in the absence of Ni^{2+}, the hydrogenase activity began to increase after a 2-h lag. The increase in activity was similar to that measured in cells which received Ni^{2+} at the start of the experiment. The increase in activity on addition of Ni^{2+} was protein synthesis dependent, since the inclusion of chloramphenicol with Ni^{2+} at 6 h completely inhibited the ability of the cells to synthesize hydrogenase. These data

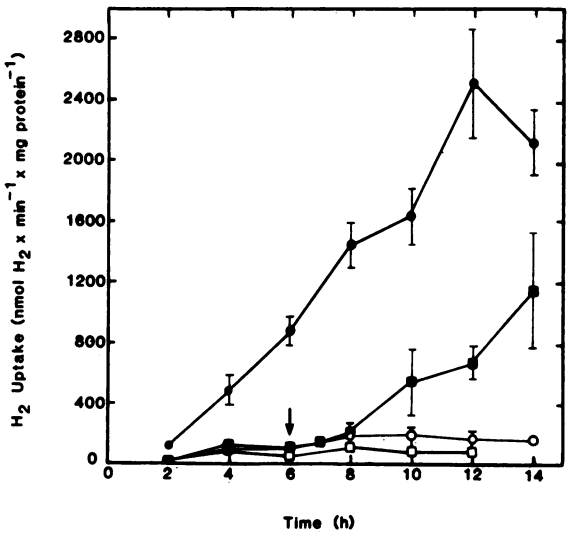

FIG. 7. Time course of the effect of Ni^{2+} addition to *A. latus* cells preinduced in the absence of Ni^{2+}. Separate cultures of *A. latus* were induced for hydrogenase activity in the absence (■, □, ○) or presence (●) of 200 nM added $NiCl_2$. After 6 h, the cultures induced in the absence of Ni^{2+} were divided into three sets. $NiCl_2$ (final concentration, 200 nM) was added to one set (■); 200 nM $NiCl_2$ and 25 μg of chloramphenicol ml^{-1} were added to the next set (□); and the last set was unaltered (○). At the indicated time points, 0.3-ml fractions were removed from each culture by syringe and the H_2 uptake activity was measured amperometrically. All treatments were performed in triplicate. Hydrogenase activities are means ± standard deviations.

suggest that added Ni^{2+} does not activate preformed hydrogenase apoprotein.

Specificity of *A. latus* hydrogenase for Ni^{2+}. The ability of selected metal ions to substitute for Ni^{2+} in the expression of hydrogenase protein and activity was tested. *A. latus* cells were induced for hydrogenase in the presence of Sr^{2+} (0.1 to 100 μM), Cd^{2+} (0.1 to 10 μM), Cr^{3+} (0.1 to 100 μM), Pb^{2+} (0.1 to 100 μM), and Sn^{2+} (0.1 to 100 μM) in place of Ni^{2+}. In no case did hydrogenase activity increase above that of controls with no added Ni^{2+} (data not shown). This is consistent with what has been observed for other hydrogen-oxidizing bacteria (7, 13, 20, 23). Also, no hydrogenase protein was detected in Western blots of crude extracts from *A. latus* cells induced in the absence of added Ni^{2+} and in the presence of these various metals (data not shown). Thus, there was no production of inactive hydrogenase containing any of the tested metal ions in place of nickel. Furthermore, none of the essential trace elements provided substituted for nickel, because in the absence of added nickel neither hydrogenase activity nor protein was detected.

DISCUSSION

We have examined the role of Ni in the expression of hydrogenase in *A. latus*. The results indicate that Ni is necessary for the expression of hydrogenase activity and protein in *A. latus*. Maximal hydrogenase activity was reached at 30 nM added Ni^{2+}. Similar experiments performed with *B. japonicum* showed that hydrogenase activity reached maximal levels at about 250 nM added Ni^{2+} (21). With both *A. latus* (see above) and *B. japonicum* (21), the amounts of hydrogenase protein, as measured by immunoblotting techniques, correlated with the levels of hydrogenase activity. Thus, in the absence of added Ni^{2+}, no hydrogenase apoenzyme was detected. In contrast, part of the soluble hydrogenase apoenzyme, containing diaphorase activity, is present in *Alcaligenes eutrophus* H16 incubated in the absence of added Ni^{2+} and in the presence of EDTA (8). Likewise, high levels of the apoenzyme of the nickel-containing carbon monoxide dehydrogenase of *Rhodospirillum rubrum* were formed in cells induced for this enzyme in the absence of nickel (2). Furthermore, the *R. rubrum* carbon monoxide dehydrogenase apoenzyme can be activated by the addition of nickel, even in the presence of chloramphenicol. This is not the case for the hydrogenase of either *B. japonicum* (21) or *A. latus* (see above).

In *B. japonicum*, the absence of hydrogenase apoenzyme on induction in Ni^{2+}-depleted medium was interpreted in terms of an effect of Ni at the transcriptional level; i.e., in the absence of Ni^{2+} hydrogenase, apoprotein is not synthesized (21). Transcriptional regulation of hydrogenase synthesis would also explain our results with *A. latus*. However, transcriptional regulation by Ni implies that a threshold Ni^{2+} concentration for hydrogenase induction exists. Even at the lowest concentrations of added Ni^{2+} tested (10 nM), hydrogenase synthesis in *A. latus* was induced, as evidenced by the presence of both hydrogenase activity and protein. It is possible that the minimal concentration of Ni^{2+} necessary to induce hydrogenase synthesis in *A. latus* was below our experimental limits. In considering the data in Fig. 5, however, it is difficult to imagine a transcriptional regulatory mechanism which gives rise to 1 nmol of hydrogenase per nmol of Ni^{2+} added to the cultures. An alternative explanation is that Ni has no role in regulating the synthesis of *A. latus* hydrogenase at the transcriptional level. Instead, hydrogenase apoprotein could still be made in the absence of nickel, but with no nickel present to stabilize the tertiary structure of the enzyme, the apoprotein would be rapidly degraded. Protein degradation in *E. coli* is a well-studied phenomenon; at least eight different proteases have been isolated (9). In *E. coli*, truncated proteins and proteins that contain amino acid replacements are rapidly and completely degraded. Almost nothing is known about proteolysis in either *A. latus* or *B. japonicum*, and it seems possible that the lack of immunoreactive material in both organisms upon Ni^{2+} deprivation is due not to transcriptional regulation by nickel, but rather to rapid degradation of apoprotein. Indeed, both processes could be occurring simultaneously. Further studies are required to distinguish between these two possibilities.

A third possible explanation for our data is that hydrogenase apoenzyme accumulates in the absence of nickel, but is not recognized by our polyclonal sera. However, as pointed out by Stults et al. for *B. japonicum*, this is unlikely for several reasons (21). In both cases, the sera used were polyclonal and were reacted against sodium dodecyl sulfate (SDS)-denatured cell extracts. The polyclonal serum used in our experiments, which was raised against purified active *A. latus* hydrogenase protein, was effective in recognizing both non-SDS-treated- and SDS-treated hydrogenase (data not shown). Accumulation of *A. latus* hydrogenase apoprotein not detectable by our polyclonal sera in SDS-treated cell extracts therefore seems unlikely.

These data provide evidence for a specific role for Ni in regulating the levels of hydrogenase protein, and thereby activity, in *A. latus*. It is presently unclear whether this effect of Ni is mediated at the transcriptional level. Direct measurement of hydrogenase mRNA levels, through the use

of a hydrogenase-specific DNA probe, should provide a clearer picture of the mechanism of Ni regulation.

Nickel incorporation in *A. latus* exhibited two distinct phases, based on the amounts of Ni^{2+} added to the cultures. At low added Ni^{2+} concentrations (<30 nM), all of the added nickel could be accounted for in hydrogenase on the basis of our estimates of the nanomolar quantities of hydrogenase in these cultures. The 1:1 relationship between added Ni^{2+} and calculated hydrogenase protein levels at limiting Ni^{2+} concentrations would indicate that there is one atom of Ni per hydrogenase molecule. This compares with the value obtained by Pinkwart et al. (19) of 0.54 mol of Ni per mol of *A. latus* hydrogenase. Consistent with the equivalence of added Ni^{2+} and calculated hydrogenase quantities is the observation that at low Ni^{2+} concentrations, all the added Ni^{2+} was associated with the cells. In the second phase, between 50 and 200 nM added Ni^{2+}, Ni^{2+} continued to accumulate in the cells, but also remained in the medium. Nickel incorporation into *A. latus* cells was saturated at 200 nM added Ni^{2+}. Because hydrogenase activity is saturated at 50 nM added Ni^{2+}, the additional Ni^{2+} accumulated by the cells between 50 and 200 nM added Ni^{2+} was probably not associated with hydrogenase. Methylene blue-dependent hydrogenase activity of broken *A. latus* cells previously induced in the presence of 50 and 100 nM added Ni^{2+} was not higher than the O_2-dependent activity, suggesting that there is not a pool of non-O_2-linked hydrogenase in the cell (data not shown). It seems likely that at least part of the Ni associated with the cells would be bound to the cell wall fraction, as has been found for other bacteria (12). However, the amount of Ni bound to Ni^{2+}-depleted cells grown heterotrophically was only 15 to 20% of the amount of Ni that induced cells bound at any given Ni^{2+} concentration (data not shown). Thus, there appears to be a sink for Ni^{2+} in *A. latus* other than hydrogenase and the cell wall.

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