JOURNAL OF BACTERIOLOGY, Oct. 1982, p. 42–48
0021-9193/82/100042-07$02.00/0

Vol. 152, No. 1

Nickel in the Catalytically Active Hydrogenase of *Alcaligenes eutrophus*

CORNELIUS G. FRIEDRICH,* KLAUS SCHNEIDER, AND BÄRBEL FRIEDRICH
Institut für Mikrobiologie der Universität Göttingen, 3400 Göttingen, Federal Republic of Germany

Received 12 April 1982/Accepted 18 June 1982

Nickel is a constituent of soluble and particulate hydrogenase of *Alcaligenes eutrophus*. Incorporation of $^{63}Ni^{2+}$ revealed that almost the total nickel taken up by the cells was bound to the protein. Chromatography of a crude extract on diethylaminoethyl cellulose demonstrated an association of $^{63}Ni^{2+}$ with soluble and particulate hydrogenase, supported by further analysis like polyacrylamide gel electrophoresis. Unspecific binding of $^{63}Ni^{2+}$ to the protein was excluded by comparison with a mutant extract free of hydrogenase protein. X-ray fluorescence analysis of the homogeneous soluble hydrogenase indicated the presence of 2 mol of nickel per mol of enzyme, whereas the amount of nickel determined by incorporation of $^{63}Ni^{2+}$ was calculated to be approximately 1 mol/mol of enzyme. Cells grown under nickel limitation contained catalytically inactive, but serologically active, soluble and particulate hydrogenase. The immunochemical reactions were only partially identical with the enzyme from nickel-cultivated cells indicating a structural modification of the proteins in the absence of nickel. It is concluded that nickel is essential for the catalytic activity of hydrogenase and not involved as a regulatory component in the synthesis of this enzyme.

Chemolithoautotrophic growth of *Alcaligenes eutrophus* with hydrogen, oxygen, and carbon dioxide specifically requires the transition metal nickel (3, 20). Growth on formate did not depend on the presence of nickel in the medium. Carbon dioxide produced from formate is assimilated via the Calvin cycle (11), suggesting that nickel is not involved in autotrophic carbon dioxide fixation, but is necessary for lithotrophic hydrogen oxidation (9).

Heterotrophic growth on fructose or glycerol was also not affected by the availability of nickel to the cells. We have shown that under specific heterotrophic conditions key enzymes of chemolithoautotrophic metabolism, such as the soluble hydrogenase (hydrogen:NAD^+ oxidoreductase, EC. 1.12.1.2) and the membrane-bound hydrogenase, are formed at a high level, although they are not essential for growth (9). Taking advantage of this derepression system, we further demonstrated that the synthesis of catalytically active hydrogenases required nickel ions. Thus it was concluded that nickel might be a constituent of the hydrogenase proteins or part of a regulatory component (9).

In this report, we present evidence that nickel is in fact a constituent of catalytically active hydrogenase protein. This was deduced from the incorporation of $^{63}Ni^{2+}$ into the enzymes, energy-dispersive X-ray fluorescence studies on the homogeneous soluble hydrogenase protein, and from immunochemical analyses.

MATERIALS AND METHODS

Chemicals. NAD^+ and DNase I were obtained from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Diethylaminoethyl (DEAE) cellulose was purchased from Whatman Biochemicals, Maidstone, U.K.; Procion red was purchased from Amicon GmbH, Witten, West Germany, and DEAE-Sephacel was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Radioactive nickel was obtained as $^{63}NiCl_2$ with a specific activity of 0.716 mCi/μmol from the Radiochemical Centre, Amersham, England. All other chemicals were purchased from E. Merck AG, Darmstadt, Germany.

Bacterial strains. *A. eutrophus* strain H16 (ATCC 17699, DSM 428) and mutants derived from this strain were used throughout this study.

Growth conditions. The bacteria were grown in mineral salts medium as described by Schlegel et al. (17) with the following modifications: no trace elements were added, and the concentration of $FeCl_3$ was 5 mg/liter. $^{63}Ni^{2+}$ was added at a concentration of 0.07 μM, if not otherwise stated. The concentration of the heterotrophic carbon sources fructose and glycerol was 0.2% (wt/vol) each. Ammonium chloride (0.2% [wt/vol]) served as the nitrogen source. Growth temperature was 30°C. Media were inoculated with 1% (vol/vol) of a culture which had been grown for 20 h on fructose-mineral medium. $^{63}Ni^{2+}$-incorporation experiments were routinely carried out in 100 ml of

medium in a 500-ml side arm flask which was incubated on a rotary shaker for 70 to 76 h. Cells were cooled rapidly to 4°C before centrifugation. They were washed once with cold 50 mM potassium phosphate buffer (pH 7.0) and stored at −20°C.

For the preparation of homogeneous soluble hydrogenase, cells were grown for 76 h in 10 liters of mineral medium containing trace elements and ferric ammonium citrate as previously described (12). Fructose and glycerol (0.2% each [wt/vol]) were added as the carbon sources.

Preparation of cell extracts. All steps were performed at 0 to 4°C. Cells grown in 100 ml of $^{63}Ni^{2+}$-containing fructose-glycerol medium were resuspended in 4 ml of 20 mM potassium phosphate buffer (pH 6.8) and subjected to ultrasonic disruption (10). Cell debris was removed by centrifugation (9). The supernatant was referred to as crude extract. The soluble and the particulate cell fractions were separated by centrifugation of the crude extract as previously described (9).

Chromatography of cell extracts on DEAE-cellulose. Approximately 3.5 ml of the crude extract (approximately 32 mg of protein) was applied to a column of DEAE-cellulose (1.5 by 20 cm) that had been equilibrated with 20 mM potassium phosphate buffer (pH 6.8). Protein was eluted with a 0 to 0.5 M linear KCl gradient prepared in 200 ml of the buffer described above. Fractions of 2 ml were collected at a flow rate of 20 ml/h.

Electrophoresis. Analysis of the native hydrogenases was done at 0 to 4°C by nondenaturing polyacrylamide gel electrophoresis (PAGE) as described (8). Peak fractions of soluble and particulate hydrogenase from chromatography on DEAE-cellulose were concentrated 5- to 10-fold with Minicon B15 concentrators (Amicon GmbH., Witten, West Germany). Samples (5 to 40 μl) were applied to disk gels containing 7.5% acrylamide. The electrophoresis, staining, and destaining was done as described previously (8). The content of radioactive nickel in the gels was done as described by Graf and Thauer (13).

Preparation of homogeneous soluble hydrogenase. The soluble hydrogenase was purified to homogeneity as described (18) with the following alterations. The ammonium sulfate precipitate was dialyzed against 50 mM potassium phosphate buffer (pH 6.2) and subsequently applied to a column of Procion red HE-3B agarose (5 by 60 cm). Protein not bound to the gel was washed off the column with dialysis buffer. Hydrogenase was eluted with a 0 to 1 M linear KCl gradient prepared in the buffer described above. Fractions containing hydrogenase activity were combined, concentrated, and dialyzed against 50 mM potassium phosphate buffer (pH 7.0). This extract was further purified by DEAE-chromatography (18). However, instead of DEAE-cellulose DEAE-Sephacel was used, and gel filtration as well as preparative gel electrophoresis were omitted.

Quantitative determination of nickel. The content of nickel in samples was determined by two procedures. Routinely, $^{63}Ni^{2+}$ was added as $NiCl_2$ to the medium at various concentrations without dilution by nonradioactive nickel ions. The content of nickel in the culture fluid, cells, and protein fraction was determined by following the radioactivity. Samples (100 μl) were added to 5 ml of Quickscint 212 (Zinsser, Frankfurt) and counted for 5 min in a Packard liquid scintillation counter.

The trace metal analysis of the homogeneous soluble hydrogenase was carried out by energy-dispersive X-ray fluorescence measurements. The new type of totally reflecting X-ray spectrometer used was previously described by Knoth and Schwenke (14). With this instrument the detection limit of about 20 metals including nickel is below 10^{-11} g per sample.

Enzyme assays. Enzyme assays were carried out at 30°C. The activity of soluble hydrogenase was determined with the soluble fraction by monitoring NADH formation spectrophotometrically by the method of Schneider and Schlegel (19). The membrane-bound hydrogenase activity was assayed by the spectrophotometric measurement of hydrogen-dependent methylene blue reduction (16). The immunochemical analysis of the soluble and the particulate hydrogenase proteins from cell extracts was done by the immunodiffusion method of Ouchterlony as previously described (10).

RESULTS

Uptake of nickel ions. The uptake of nickel(II) ions by *A. eutrophus* was determined with radioactive $^{63}Ni^{2+}$ (Fig. 1). The uptake of nickel followed biphasic kinetics. Approximately 15% was taken up during exponential growth on fructose. During the substrate downshift to glycerol (9), there was a large increase of nickel uptake which coincided with the increase of soluble hydrogenase activity (Fig. 1) and the membrane-bound hydrogenase activity (data not shown). The rate of nickel uptake during the period of active hydrogenase formation decreased with decreasing initial concentrations of nickel chloride (data not shown).

Further evidence for a correlation of nickel uptake with the formation of active hydrogenase

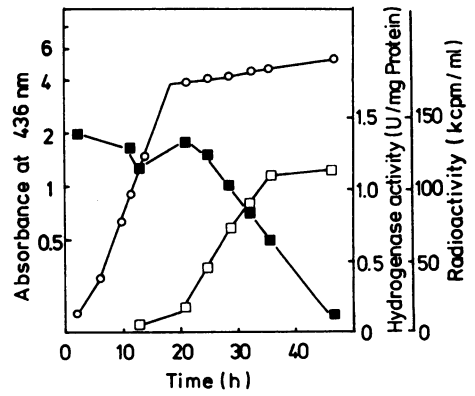

FIG. 1. Nickel uptake during growth and hydrogenase formation of *A. eutrophus*. Cells were grown in 100 ml of fructose-glycerol mineral medium. $^{63}NiCl_2$ (1 μM; 0.7 μCi/ml) was added before inoculation. The radioactive nickel (■) was determined from 100 μl of the clear supernatant after harvest. Soluble hydrogenase activity (□) was determined from whole cells. growth is presented in a semilogarithmic plot (○).

TABLE 1. Uptake of nickel by mutants of *A. eutrophus*

Strain	Phenotype[a]	Residual nickel[b] (%)
H16	Hox^+	1.7
HF08	Hop^-	1.9
HF14	Hos^-	7.6
HF18	Hox^-	63.2

[a] Hox^+, ability to form active soluble and membrane-bound hydrogenase; Hop^-, inability to form active membrane-bound hydrogenase; Hos^-, inability to form active soluble hydrogenase; and Hox^-, inability to form active soluble and membrane-bound hydrogenase.

[b] Cells were grown for 72 h in 100 ml of fructose-glycerol mineral medium supplemented with 0.07 μM $^{63}Ni^{2+}$. The cells were collected by centrifugation. The radioactivity was determined from the culture fluid after removal of the cells and was related to the initial radioactivity set as 100%.

was obtained with mutants impaired in the activities of either the soluble or the membrane-bound hydrogenase or both (Table 1). The uptake of nickel by the parent strain H16 was 98% of that amount initially present in the medium. No alteration of nickel uptake was observed with the mutant HF08 which is unable to form active membrane-bound hydrogenase (15). Distinctly less nickel (92%) was taken up by strain HF14 defective in the activity of soluble hydrogenase (10). However, only 37% of the nickel ions were taken up by the Hox^- mutant HF18, which is impaired in the synthesis of both hydrogenase proteins (10).

Localization of nickel in cell-free extracts. Extracts of cells grown in the presence of $^{63}Ni^{2+}$ were treated with DNase and RNase and subsequently used to separate low-molecular-weight compounds from proteins by gel filtration and dialysis. A total of 96% of the radioactive nickel was recovered in the protein fraction after chromatography of the crude extract on Sephadex G-25. Only traces of $^{63}Ni^{2+}$ were eluted with the salt fraction. After a 27-h dialysis of the crude extract against buffers of pH from 6.0 to 7.6, only 9.5 to 12.7% of the total radioactivity was found in the dialysis buffer.

Since the crude extract contained both the soluble and the particulate protein fractions, we investigated whether the radioactive nickel was associated with either one fraction or with both. Approximately 80% of the radioactivity was located in the soluble fraction, whereas 20% of the radioactive nickel was present in the particulate fraction. The soluble extract contained the NAD-reducing hydrogenase, whereas most of the membrane-bound enzyme resided in the particulate fraction. However, a minor part of the latter enzyme dissociated from the membrane during ultrasonic treatment of the cells as shown by immunochemical analysis (see Fig. 4B).

Distribution of $^{63}Ni^{2+}$ during partial purification of hydrogenase. A crude extract derived from cells which had been grown in a $^{63}Ni^{2+}$-supplemented fructose-glycerol minimal medium was subjected to chromatography on DEAE-cellulose. (Fig. 2B). The first peak of radioactive nickel was eluted with proteins which did not adsorb to the cellulose. A small second peak was eluted with traces of particulate hydrogenase activity representing parts of the enzyme which had been dissociated from the membrane during extract preparation. The enzyme was clearly

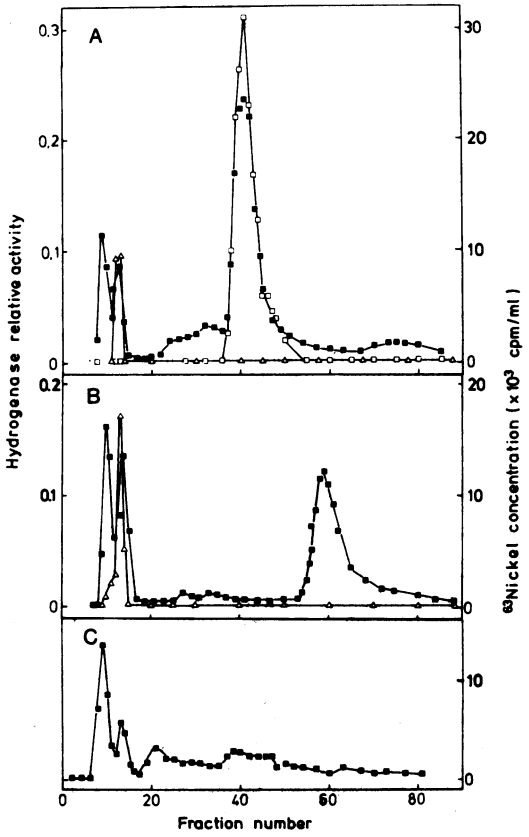

FIG. 2. Chromatography of extracts of *A. eutrophus* on DEAE-cellulose. About 3.5 ml of crude extracts from cells of strain H16 (A), strain HF14 (B), and strain HF18 (C) grown in 100 ml of fructose-glycerol mineral medium in the presence of 0.07 μM $^{63}Ni^{2+}$ (0.05 μCi/ml) were subjected to chromatography on DEAE-cellulose. From the eluate fractions, 100 μl was used to determine radioactive nickel (■). For determination of the particulate hydrogenase (△), 100 μl was used, and 50 μl was used for the soluble hydrogenase activity (□).

identified as such by a hydrogen-dependent methylene blue reduction at a pH optimum of 5.5 and being inactive with NAD as the electron acceptor (16). A major third peak of $^{63}Ni^{2+}$ contained 55% of the totally recovered nickel and exactly coincided with the elution profile of soluble hydrogenase activity. The total recovery of $^{63}Ni^{2+}$ applied to the column was 83%.

Nickel is known to bind unspecifically to proteins (13). To exclude a nonspecific association of nickel with hydrogenase, we subjected extracts of mutants impaired in either the catalytic activity of soluble hydrogenase (Hos^-) or the synthesis of both hydrogenase proteins (Hox^-) to chromatography on DEAE-cellulose. With the extract of the Hos^- mutant HF14 again three peaks of radioactive $^{63}Ni^{2+}$ were observed (Fig. 2B). The first two peaks were identical with the elution profile of the wild-type extract. The third major peak representing 53% of the totally recovered nickel (88%) appeared at a higher potassium chloride concentration than the wild-type enzyme. However, immunochemical analysis revealed a precipitate of the concentrated peak fractions with purified antisoluble hydrogenase serum (data not shown). This was a clear evidence for the presence of serologically cross-reacting but catalytically inactive soluble hydrogenase protein in this mutant. On the other hand, crude extracts from the Hox^- mutant HF18 proved to be antigenically inactive with antiserum of purified soluble hydrogenase from the parent strain H16 (10). Thus, this mutant appeared to be suitable for the examination of unspecific binding of $^{63}Ni^{2+}$ to proteins. In fact, the first peak of radioactivity coincided with the peak observed in the preceding experiments. The second peak was distinctly smaller, whereas the rest of the radioactive nickel was nonpreferentially distributed in the eluate (Fig. 2C). The total recovery of $^{63}Ni^{2+}$ was 84%.

The partially purified soluble hydrogenase obtained by chromatography on DEAE-cellulose was further subjected to filtration on Sephadex G-200 and sucrose gradient centrifugation. In both experiments, the radioactive nickel was associated with hydrogenase activity (data not shown), indicating that binding of $^{63}Ni^{2+}$ was not random.

Soluble and ultrasonically solubilized particulate hydrogenase of partially purified preparations derived from chromatography on DEAE-cellulose were subjected to nondenaturating polyacrylamide gel electrophoresis (PAGE) (8). Soluble hydrogenase migrated with radioactive nickel in one peak (Fig. 3A). A broad small peak of $^{63}Ni^{2+}$ was observed during PAGE of the particulate hydrogenase (Fig. 3B). This enzyme is known to split into two bands of activity during PAGE (16).

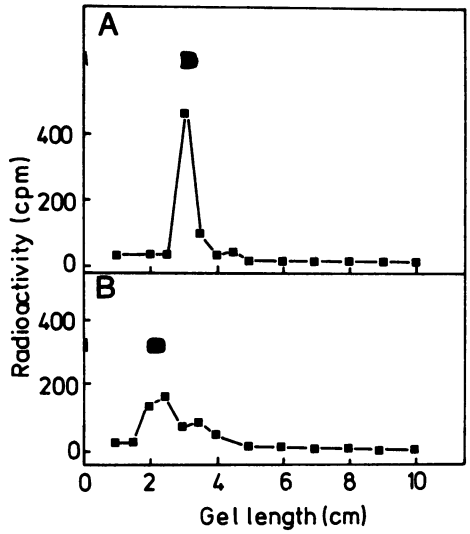

FIG. 3. Chromatography of soluble and particulate hydrogenase by PAGE. Each 40 μl of partially purified preparations of soluble (A) and particulate hydrogenase (B) (for details, see the text) were applied to 7.5% polyacrylamide gels (0.6 by 10.5 cm) and chromatographed for 2 h at 4 mA per gel. Radioactive nickel was determined as described previously (13). About 12 μg of almost homogeneous soluble and particulate hydrogenase was used as reference proteins and was stained and destained as described previously (8).

Nickel content of soluble hydrogenase. The nickel content of the soluble hydrogenase was determined by specific incorporation of radioactive nickel into the hydrogenase protein and by direct determination of nickel of the homogeneous protein by X-ray fluorescence.

The first method resulted in a calculated nickel content of 0.87 mol/mol of enzyme. For this determination, fraction 41 of Fig. 2A was used. Since the contamination level of the medium with nonradioactive nickel was unknown, X-ray fluorescence analysis was applied as a second independent method.

The soluble hydrogenase was purified to homogeneity from cells grown with nonradioactive $^{58}NiCl_2$ as described above. The purification was performed by an improved procedure, including affinity chromatographay on Procion red agarose as an essential step (K. Schneider, submitted for publication).

The final specific activity of the homogeneous enzyme was 90 U/mg of protein. The enzyme preparation was analyzed for the content of nickel by energy dispersive X-ray fluorescence (14). Other metals, such as iron and copper, were included as references. The nickel content of the enzyme increased linearly with increasing

TABLE 2. Nickel content of homogeneous soluble hydrogenase

Hydrogenase concn[a] (mg/ml)	Metal content (mol/mol of hydrogenase)		
	Nickel	Iron	Copper
1.05	1.89	15.6	0.12
2.10	1.99	15.9	0.17
4.20	2.07	16.2	0.11

[a] Homogeneous hydrogenase (0.5 ml; 5 mg/ml) was dialyzed against 10 mM Tris-hydrochloride (pH 7.5) and adjusted to various protein concentrations as indicated. Samples of 20 μl were analyzed for metal content by X-ray fluorescence (14).

protein concentration of the samples (data not shown). This was further evidence for nickel being a component of hydrogenase. On the basis of a molecular weight of 205,000 (19), a nickel content of 2 mol/mol of enzyme was calculated. The determination of the iron content of the enzyme by X-ray fluorescence revealed 16 mol/mol of enzyme. This result was in good agreement with earlier data obtained during the characterization of the iron-sulfur centers of the soluble hydrogenase from *A. eutrophus* (18).

No evidence is available for the involvement of other metals than iron and nickel in the formation of active hydrogenase. Traces of copper of approximately 0.11 to 0.17 mol/mol of enzyme were detected without a strict correlation to the protein concentration (Table 2). Other metals, such as zinc, manganese, and cobalt, were found in similar or even lower quantities (data not shown), indicating a nonspecific binding to the enzyme.

Nickel-dependent serological and catalytic activity of the hydrogenases. In a previous communication, we have shown that the omission of nickel from the medium did not affect heterotrophic growth in fructose-glycerol minimal medium but resulted in a significant decrease of soluble and particulate hydrogenase activity. Nickel limitation was achieved by the addition of the chelating agent EDTA. Attempts to reactivate hydrogenase by adding nickel to a crude extract derived from cells which had been grown with fructose-glycerol in an EDTA-containing medium were unsuccessful. Thus it was suggested that nickel may be involved in a protein synthesis-dependent process (9). We reexamined this point by analyzing extracts from cells grown in the presence and absence of nickel for catalytic and serological activity of soluble and particulate hydrogenase. Mutants defective in either one of the two enzymes were included in this study (Table 3). There was no catalytic activity of soluble hydrogenase in the presence of EDTA. However, there was immunologically cross-reacting material detected with antiserum of purified soluble hydrogenase from *A. eutrophus*. A comparison of the immunological reaction with the corresponding nickel-containing extract revealed that nickel limitation led to the occurrence of a faster moving precipitation band of partial identity (Fig. 4A), indicating a structural alteration of the enzyme molecule. These changes were also visible with an extract of the Hos^- mutant HF14 lacking catalytically active soluble hydrogenase but exhibiting partial serological activity (Table 3). The catalytic activity of the particulate hydrogenase was also strictly nickel dependent; however, the formation of immunologically cross-reacting material apparently was not nickel dependent (Table 3). Under

TABLE 3. Immunochemical analysis of extracts from nickel-limited cells

Strain	Phenotype[a]	EDTA[b] (μM)	Hydrogenase activity[c]			
			Catalytic		Serological	
			SH	PH	SH	PH
H16	Wild-type	0	2.20	0.78	CRM^+	CRM^+
		10	0.07	0.01	CRM^P	CRM^+
HF08	Hop^-	0	2.40	0	CRM^+	CRM^+
		10	0.06	0	CRM^P	CRM^+
HF14	Hos^-	0	0	0.59	CRM^P	CRM^+
		10	0	0.01	CRM^P	CRM^+

[a] Hop^-, impaired in particulate hydrogenase activity; Hos^-, impaired in soluble hydrogenase activity.

[b] The cells were grown in fructose-glycerol minimal medium with 8 μM $NiCl_2$. When EDTA was present in the medium, no nickel was added.

[c] The catalytic activity of soluble (SH) and particulate (PH) hydrogenase is expressed as units per milligram of protein. The serological activity was determined by agar gel immunodiffusion (Ouchterlony) with antisoluble and antiparticulate hydrogenase serum. CRM^+, cross-reaction identical with the respective purified enzyme; CRM^P, cross-reaction of partial identity.

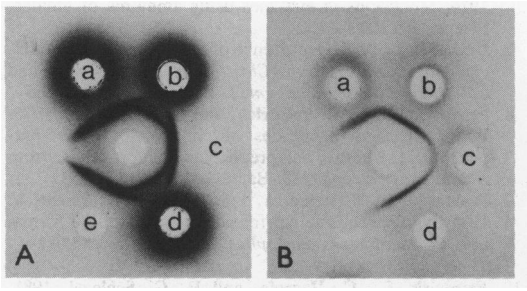

FIG. 4. Immunodiffusion analysis of soluble and particulate hydrogenase formation by *A. eutrophus* H16. Extracts were analyzed for the presence of hydrogenase as described (9). The center well of gel A contained purified antisoluble hydrogenase serum. Outer wells contained soluble extracts of cells grown with fructose-glycerol in nickel-containing medium (a, c, and e) and a nickel-limited medium with EDTA (b and d). The center well of gel B contained antiparticulate hydrogenase serum. Solubilized particulate extract (16) of nickel-grown cells (a) and nickel-free cells (c) was applied to the outer wells in addition to the corresponding soluble extracts b and d.

nickel limitation, virtually all of the serological activity of the membrane-bound hydrogenase resided in the soluble fraction instead of being distributed on both the soluble and the particulate fraction (Fig. 4B). This suggests that under nickel starvation, the enzyme is less tightly bound to the membrane, presumably as a result of a structural modification.

DISCUSSION

Nickel is an essential component of catalytically active hydrogenase from *A. eutrophus*. This conclusion is based on the following observations. (i) Virtually all of the nickel incorporated was present in the protein fraction. (ii) Chromatography of crude extracts on DEAE-cellulose resulted in a comigration of $^{63}Ni^{2+}$ with soluble and particulate hydrogenase activities. (iii) Binding of $^{63}Ni^{2+}$ was not random, since the major peak of radioactivity was absent in the eluate of a mutant extract impaired in the synthesis of hydrogenase (10). (iv) Further purification of the soluble hydrogenase by gel filtration, sucrose gradient centrifugation, and PAGE clearly established a specific association of nickel with the hydrogenase proteins. In addition to the biochemical data, physiological evidence was obtained for the particulate hydrogenase. Strain HF14 unable to form an active soluble enzyme grows autotrophically with hydrogen only in the presence of nickel ions. (v) Energy-dispersive X-ray fluorescence studies of homogeneous soluble hydrogenase indicated the presence of 2 mol of nickel per mol of enzyme compared with 0.87 mol calculated from incorporation of $^{63}Ni^{2+}$.

Nickel is essential for autotrophic growth of *A. eutrophus* (3), and Ni^{2+} uptake is an energy-requiring process in this strain (20). Recently evidence accumulated showing that nickel is involved in several metabolic processes. First, urease was reported to contain nickel tightly bound to the enzyme (6). Carbon monoxide dehydrogenase from acetogenic bacteria appeared to be a nickel protein (5, 7). Nickel occurred to be a constituent of factor F_{430} present in methanogenic bacteria (4). Finally, a further nickel-containing cell component was identified in this group of organisms, i.e., the hydrogenase from *Methanobacterium thermoautotrophicum* (13). On the other hand, no experimental evidence was obtained for the involvement of nickel in the formation of active hydrogen evolving hydrogenase from *Clostridium pasteurianum* (7). Conversely, the activity of the membrane-bound uptake hydrogenase from the photosynthetic bacterium *Rhodopseudomonas capsulata* was significantly stimulated by nickel in the medium (21). Attempts to enhance this hydrogenase activity by adding nickel to a cell-free extract were unsuccessful, as similarly described for *A. eutrophus* (9).

A stimulating effect of nickel on the in vitro activity of hydrogenase has been reported on the soluble NAD-reducing enzyme from the gram-positive bacterium *Nocardia opaca* 1b (1). However, this stimulation required a relatively high concentration of nickel (1 mM) and was not strictly specific, since the same effect was achieved with high salt concentrations (0.5 to 2 M; K. Schneider, unpublished results). These observations do not exclude a specific nickel requirement for chemolithoautotrophic growth of *N. opaca* as described by Tabillion et al. (20). According to recent growth experiments, growth with hydrogen as the sole energy source was indeed nickel dependent (M. Reh, unpublished results).

The fact that cells of *A. eutrophus* grown under nickel limitation made serologically active soluble and particulate hydrogenase clearly restricts the function of nickel to the catalytic activity of the enzyme and excludes the possibility that nickel is involved in a regulatory protein synthesis-dependent process. Evidence for a structural alteration of the hydrogenases in the absence of nickel was presented by immunochemical analyses in comparison with the catalytically active hydrogenases.

Very recently, Albracht et al. (2) described a specific electron paramagnetic resonance signal of purified hydrogenase from *M. thermoautotrophicum* which proved to be due to nickel. Since this signal disappeared on the reduction with

molecular hydrogen and no additional electron paramagnetic resonance signal of an iron-sulfur cluster was detected, the authors suggest that nickel may be the primary site of the enzyme for hydrogen activation. The electron paramagnetic resonance properties of the soluble hydrogenase of *A. eutrophus* have been described in detail (18). However, no evidence was found for the existence of a nickel signal. Nickel might be present in this enzyme in an electron paramagnetic resonance silent form. This could be due to either a strong magnetic interaction with an iron-sulfur cluster or to the remaining of nickel in a physiologically nonoxidized (II) state during the aerobic enzyme preparation or ferricyanide treatment. In nickel complexes, nickel(II) is known to be the most stable redox state under aerobic conditions (2).

ACKNOWLEDGMENTS

The help in X-ray fluorescence studies by J. Knoth and H. Schwenke and the technical assistance by S. Erfurth during part of this work is thankfully acknowledged.

This study was supported by a grant of the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Aggag, M., and H. G. Schlegel.** 1974. Studies on a gram-positive hydrogen bacterium, *Nocardia opaca* 1b. Arch. Microbiol. **100**:25–39.
2. **Albracht, S. P. J., E.-G. Graf, and R. K. Thauer.** 1982. The EPR properties of nickel in hydrogenase from *Methanobacterium thermoautotrophicum*. FEBS Lett. **140**:311–313.
3. **Bartha, R., and E. J. Ordal.** 1965. Nickel-dependent chemolithoautotrophic growth of two *Hydrogenomonas* strains. J. Bacteriol. **89**:1015–1019.
4. **Diekert, G., B. Klee, and R. K. Thauer.** 1980. Nickel, a component of factor F_{430} from *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **124**:103–106.
5. **Diekert, G. B., and R. K. Thauer.** 1980. The effect of nickel on carbon monoxide dehydrogenase formation in *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. FEMS Microbiol. Lett. **7**:187–189.
6. **Dixon, N. E., C. Gazzola, R. L. Blakeley, and B. Zerner.** 1976. Metal ions in enzymes using ammonia or amides. Science **191**:1144–1150.
7. **Drake, H. L.** 1982. Occurrence of nickel in carbon monoxide dehydrogenase from *Clostridium pasteurianum* and *Clostridium thermoaceticum*. J. Bacteriol. **149**:561–566.
8. **Friedrich, B., C. G. Friedrich, and H. G. Schlegel.** 1976. Purification and properties of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase from *Alcaligenes eutrophus*. J. Bacteriol. **126**:712–722.
9. **Friedrich, B., E. Heine, A. Finck, and C. G. Friedrich.** 1981. Nickel requirement for active hydrogenase formation in *Alcaligenes eutrophus*. J. Bacteriol. **145**:1144–1149.
10. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147**:198–205.
11. **Friedrich, C. G., B. Bowien, and B. Friedrich.** 1979. Formate and oxalate metabolism in *Alcaligenes eutrophus*. J. Gen. Microbiol. **115**:185–192.
12. **Friedrich, C. G., B. Friedrich, and B. Bowien.** 1981. Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*. J. Gen. Microbiol. **122**:69–78.
13. **Graf, E.-G., and R. K. Thauer.** 1982. Hydrogenase from *Methanobacterium thermoautotrophicum*, a nickel-containing enzyme. FEBS Lett. **136**:165–169.
14. **Knoth, J., and H. Schwenke.** 1980. A new totally reflecting X-ray fluorescence spectrophotometer with detection limits below 10^{-11} g. Z. Anal. Chem. **301**:7–9.
15. **Schink, B., and H. G. Schlegel.** 1978. Mutants of *Alcaligenes eutrophus* defective in autotrophic metabolism. Arch. Microbiol. **117**:123–129.
16. **Schink B., and H. G. Schlegel.** 1979. The membrane-bound hydrogenase of *Alcaligenes eutrophus*. Biochim. Biophys. Acta **567**:315–324.
17. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien. Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38**:209–222.
18. **Schneider, K., R. Cammack, H. G. Schlegel, and D. O. Hall.** 1979. The iron-sulfur centres of soluble hydrogenase from *Alcaligenes eutrophus*. Biochim. Biophys. Acta **578**:445–461.
19. **Schneider, K., and H. G. Schlegel.** 1976. Purification and properties of soluble hydrogenase from *Alcaligenes eutrophus* H16. Biochim. Biophys. Acta **452**:66–80.
20. **Tabillion, R., F. Weber, and H. Kaltwasser.** 1980. Nickel requirement for chemolithoautotrophic growth in hydrogen-oxidizing bacteria. Arch. Microbiol. **124**:131–136.
21. **Takakuwa, S., and J. D. Wall.** 1981. Enhancement of hydrogenase activity in *Rhodopseudomonas capsulata* by nickel. FEMS Microbiol. Lett. **12**:359–363.