Nickel Requirement for Active Hydrogenase Formation in *Alcaligenes eutrophus*

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The nickel-dependent chemolithoautotrophic growth of *Alcaligenes eutrophus* is apparently due to a requirement of nickel for active hydrogenase formation. Cells grown heterotrophically with fructose and glycerol revealed a specific activity of soluble and membrane-bound hydrogenase which was severalfold higher than the normal autotrophic level. The omission of nickel from the medium did not affect heterotrophic growth, but the soluble hydrogenase activity was reduced significantly. In the presence of ethylenediaminetetraacetic acid (EDTA), almost no hydrogenase activity was detected. The addition of nickel allowed active hydrogenase formation even when EDTA was present. When chloramphenicol was added simultaneously with nickel to an EDTA-containing medium, almost no hydrogenase activity was found. This indicates that nickel ions are involved in a process which requires protein synthesis and not the direct reactivation of a preformed inactive protein. The formation of the membrane-bound hydrogenase also appeared to be nickel dependent. Autotrophic CO_2 assimilation did not specifically require nickel ions, since formate was utilized in the presence of EDTA and the activity of ribulosebisphosphate carboxylase was not affected under these conditions.

In 1962, Bartha (1) showed that two facultatively chemolithoautotrophic strains of *Alcaligenes eutrophus,* H1 and H16, required trace elements for autotrophic growth. Later, it was found that although iron was the only heavy-metal ion essential for heterotrophic metabolism, cells grown autotrophically with hydrogen and carbon dioxide required nickel in addition to iron. Other transition metals such as copper, cobalt, zinc, and manganese could not substitute for nickel (2). Recently, these observations were confirmed and extended to other hydrogen-oxidizing bacteria such as *Xanthobacter autotrophicus, Pseudomonas flava,* and *Arthrobacter* sp. strains 11X and 12X. However, no significant nickel requirement could be ascribed to *Paracoccus denitrificans* and *Nocardia opaca* 1b (15). So far, the function of nickel in autotrophic metabolism has not been identified.

In the course of characterizing mutants of *A. eutrophus* which were unable to oxidize molecular hydrogen, we had to design heterotrophic growth conditions that allowed an optimum expression of both the soluble NAD^+-reducing hydrogenase (hydrogen:NAD^+ oxidoreductase, EC 1.12.1.2) and the membrane-bound hydrogenase activity. During these studies, we observed that heterotrophic growth of *A. eutro-*phus in a minimal medium containing fructose and glycerol as carbon sources was not affected by the availability of nickel; however, the activity of the soluble hydrogenase was significantly lower when the cells were cultivated under nickel limitation. Evidence is presented that nickel is an essential factor in the formation of active hydrogenases in *A. eutrophus.*

MATERIALS AND METHODS

Chemicals. NAD^+ and DNase I were obtained from Boehringer, Mannheim, Germany. Ribulose bisphosphate was purchased from Sigma Chemical Co., St. Louis, Mo. Sodium [^{14}C]bicarbonate was a product of Radiochemical Centre, Amersham, England. All other chemicals were purchased from E. Merck AG, Darmstadt, Germany.

Growth conditions. *A. eutrophus* strain H16 (ATCC 17699, DSM 428) was grown in the minimal salts medium described by Schlegel et al. (12) with the following modifications. No trace element solution was added, and the concentration of ferric ammonium citrate was 5 mg/liter of medium. EDTA adjusted to a pH of 7.0 was employed as a chelating agent at a concentration of 10 μM, if not otherwise stated. Nickel chloride was added as indicated. The concentration of the heterotrophic carbon source such as fructose or glycerol was 0.2% (wt/vol). Ammonium chloride (0.2%, wt/vol) served as the nitrogen source. The gas atmosphere for autotrophic growth contained a mixture of

hydrogen, oxygen, and carbon dioxide in a ratio of 8:1:1 (vol/vol).

Experiments were routinely carried out with a 100-ml culture in a 500-ml side-arm flask. A preculture (1 ml) grown for about 20 h in fructose-minimal medium served as the inoculum. Cells were incubated at 30°C on a rotary shaker. Growth was monitored with a Klett-Summerson colorimeter equipped with a 520- to 580-nm filter. One hundred Klett units was equivalent to an optical density of 1.0 measured at 436 nm with a Zeiss PL4 spectrophotometer.

Preparation of soluble and particulate cell fractions. All steps were performed at 0 to 4°C. Cells were harvested by centrifugation, washed once with 50 mM Tris-hydrochloride (pH 7.6), and resuspended in 6 ml of the same buffer (about 1 g, wet weight). DNase I (0.5 mg) was added, and the cells were disrupted in a precooled French press at 140 MPa. Cell debris was removed by centrifugation at 5,000 × g for 20 min. The supernatant was subjected to a second centrifugation at 90,000 × g for 45 min. The resulting supernatant was referred to as the "soluble fraction," to which ferricyanide was added at a final concentration of 0.5 mM to stabilize the hydrogenase. The precipitate of the second centrifugation containing the membranes was homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 7.0) and subjected to another centrifugation at 90,000 × g for 45 min to dilute the contaminating soluble hydrogenase. The resulting precipitate was homogenized as described above and referred to as the "particulate fraction."

Enzyme assays. Enzyme assays were carried out at 30°C. The soluble hydrogenase (EC 1.12.1.2) was routinely assayed with whole cells. A reaction mixture of 1 ml contained 0.95 ml of 50 mM Tris-hydrochloride (pH 8.0) saturated with H_2 and 10 μl of 0.5% (wt/vol) hexadecyltrimethylammonium bromide at 30°C. The cell suspension (15 μl; 30 to 60 μg of protein) was preincubated in this mixture for 1 min to render the cells permeable. The reaction was initiated by the addition of 25 μl of 48 mM NAD, and NADH formation was measured spectrophotometrically at 365 nm. Hydrogenase in cell extracts was determined by the method of Schneider et al. (13). The membrane-bound hydrogenase of the particulate fraction was assayed anaerobically under hydrogen by monitoring the reduction of methylene blue as described previously (10). Ribulosebisphosphate carboxylase (EC 4.1.1.39) activity was determined by ribulose bisphosphate-dependent incorporation of $^{14}CO_2$ into acid-stable products as described by Bowien et al. (3).

RESULTS

Derepression of soluble and membrane-bound hydrogenase under heterotrophic growth conditions. There are several reports on the formation of hydrogenases under heterotrophic conditions of growth (11). Recently, it has been documented that there is a correlation between the quality of the heterotrophic substrate as a carbon and energy source and the level of hydrogenase activity in cells of A. eutrophus (C. G. Friedrich, B. Friedrich, and B. Bow-

ien, J. Gen. Microbiol., in press). Fructose is known to be a mediocre carbon source for this bacterium. It enables the cells to grow with a doubling time of 145 min, and the specific activity of soluble hydrogenase is approximately 0.2 U/mg of protein. Glycerol represents an extremely poor carbon source, supporting growth of about one doubling per 8 h. However, hydrogenase activity in these slow-growing cells was found to be 0.5 U/mg of protein, which is equivalent to the activity found in cells growing autotrophically with hydrogen, oxygen, and carbon dioxide.

In view of these results, cells of A. eutrophus were grown in a minimal medium containing a mixture of two carbon sources, 0.2% fructose and 0.2% glycerol. Cells grew with fructose to an optical density of 4.0 (Fig. 1). Growth was then significantly retarded, indicating a substrate down-shift to glycerol. This transition was accompanied by an enormous increase in soluble and membrane-bound hydrogenase activity (Fig. 1). The former enzyme reached a specific activity of 4 U/mg of protein, which was almost 10-fold higher than the autotrophic level. The membrane-bound hydrogenase was derepressed coordinately to a specific activity of 2 U/mg of protein. The extremely high hydrogenase activity under heterotrophic growth conditions allowed us to investigate the function of nickel in autotrophic metabolism independently of autotrophic growth conditions.

Stimulation of soluble hydrogenase activity by nickel. Cells cultivated in a minimal medium supplemented with iron but with and without additional nickel chloride under an atmosphere of hydrogen, oxygen, and carbon dioxide grew with the same doubling time of 200 min. The specific activity of soluble hydrogenase was determined as 0.6 and 0.46 U/mg of protein, respectively. Apparently, traces of nickel present as a contaminant in the nonsupplemented medium were sufficient to support normal autotrophic growth.

When the cells were grown heterotrophically with fructose and glycerol as carbon sources (Fig. 1) and nickel was omitted from the medium, soluble hydrogenase reached only 50% of the activity for cells grown in a nickel-supplemented medium. This result was the first experimental evidence for a correlation between the nickel requirement and the presence of hydrogenase activity in the culture.

Among other metal ions tested, only nickel stimulated the hydrogenase activity (Table 1). The addition of cobalt, copper, or manganese resulted only in a low basal activity which equaled that of the nonsupplemented control.
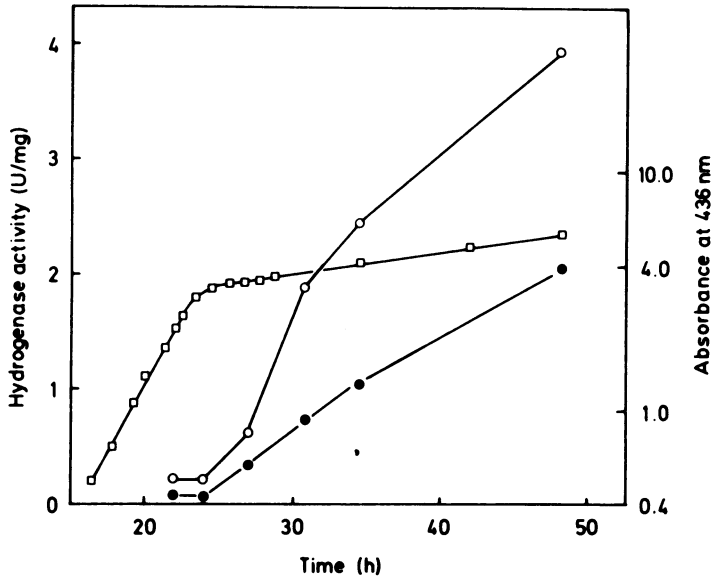
FIG. 1. *Hydrogenase activity in cells of A. eutrophus grown with fructose and glycerol as carbon sources. Cells were grown in minimal salts medium with 0.2% (wt/vol) fructose and 0.2% (wt/vol) glycerol. The growth curve is presented as a semilogarithmic plot (□). Samples were taken as indicated; soluble and particulate cell fractions were prepared to determine the soluble hydrogenase activity (○) and the membrane-bound hydrogenase activity (●).*

TABLE 1. *Specificity of nickel ions on the stimulation of hydrogenase activity*

Addition[a]	Hydrogenase activity[b]
None	0.682
Nickel	2.92
Cobalt	0.803
Copper	0.945
Manganese	0.825

[a] Chloride salts of the divalent metals were added at a concentration of 12 μM to fructose-glycerol-minimal medium.

[b] Soluble hydrogenase activity of whole cells is expressed as units per milligram of protein and was determined after 48 h of growth.

Such other divalent cations as barium, zinc, and molybdate were also ineffective (data not shown).

Soluble hydrogenase in cells grown in the presence of chelating agents. Instead of employing laborious and inefficient purification procedures to render the medium nickel-free, we added EDTA as a chelating agent. Chelators of divalent cations have been reported to inhibit the uptake of nickel into soybean cells (8) and cells of *Clostridium pasteurianum* (5). Recently, it has been documented that EDTA inhibits autotrophic growth of several hydrogen-oxidizing bacteria (15).

In the presence of 10 μM EDTA without the addition of nickel, growth in the fructose-glyc-
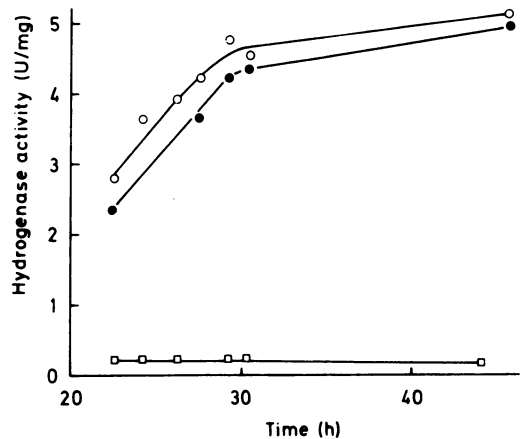

FIG. 2. *Effect of EDTA on the expression of soluble hydrogenase activity. Cells were grown as described in the legend to Fig. 1. One culture without nickel chloride contained 10 μM EDTA (□); to the other culture 8 μM nickel chloride was supplied in addition to 10 μM EDTA (●). The control contained no EDTA but 0.8 μM nickel chloride (○). Soluble hydrogenase activity was determined by the whole-cell assay.*

erol medium paralleled that of the EDTA-free control (data not shown); however, only 4% of the hydrogenase activity was detected (Fig. 2). On the other hand, when the medium was supplemented, in addition to EDTA, with 8 μM nickel, hydrogenase activity almost reached the

high, derepressed level.

The other chelating agents listed in Table 2 did not affect the expression of hydrogenase activity as much as EDTA. However, the addition of 1 mM nitrilotriacetate resulted in only 16% of the activity of the chelator-free control. In general, the presence of hydrogenase activity strictly depended on the concentration of the chelator and of nickel in the medium. The inhibition of active hydrogenase formation could be overcome by increasing the concentration of nickel in the medium (Fig. 3). There was a distinct optimum, since higher concentrations of nickel were toxic to the cells.

Attempts to activate hydrogenase from cells grown in fructose-glycerol medium containing EDTA but no nickel were unsuccessful. The

TABLE 2. Soluble hydrogenase activity in cells of A. eutrophus after growth in the presence of chelating agents

Chelating agent	Concn (mM)	Hydrogenase activity[a] (%)
None		100
EDTA	0.01	4
Nitrilotriacetate	0.10	54
	1.00	16
Citrate	1.00	82
L-Cysteine	1.00	75
L-Histidine	10.00	42

[a] Chelating agents were added at the indicated concentrations. Cells were grown in a fructose-glycerol-containing medium for 32 h. Hydrogenase activity was determined by the whole-cell assay. The chelator-free control had a specific activity of 0.64 U/mg of protein and was set as 100% activity.

addition of nickel to a soluble extract derived from these cells did not enhance hydrogenase activity. Furthermore, the addition of EDTA to an extract containing high hydrogenase activity did not inhibit the enzyme, thus showing that EDTA did not directly affect the catalytic reaction.

Effect of nickel under protein synthesis inhibition. To gain more experimental support for the hypothesis that nickel is required for active hydrogenase formation, the kinetics of hydrogenase activity were examined under conditions of protein synthesis inhibition. Cells grown in a fructose-glycerol-EDTA-containing medium were supplied with nickel, and concomitantly chloramphenicol was added. In contrast to the chloramphenicol-free control, hydrogenase activity remained at the initial low level (Fig. 4). When, instead of chloramphenicol, rifampin was added, hydrogenase activity was only enhanced by 25% (data not shown). These results strongly suggest that the stimulation of hydrogenase activity by nickel is due to its function in the synthesis of an active hydrogenase protein.

Expression of other key enzymes of autotrophic metabolism in nickel-deficient cells. Soluble hydrogenase represents only one key enzyme of autotrophic metabolism. Since mutants impaired in the soluble hydrogenase were still able to grow autotrophically, owing to the function of the membrane-bound hydrogenase (B. Friedrich, unpublished data), the question was raised whether the latter enzyme and the enzymes of the autotrophic carbon dioxide fixation were also affected by nickel. Ribulose-
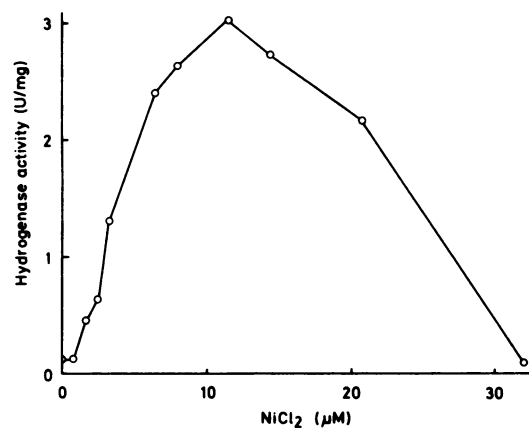

FIG. 3. Hydrogenase activity in cells grown with EDTA and various concentrations of nickel chloride. Cells were grown for 32 h as described in the legend to Fig. 1. The medium contained 10 μM EDTA and nickel chloride as indicated. Soluble hydrogenase was determined by the whole-cell assay.
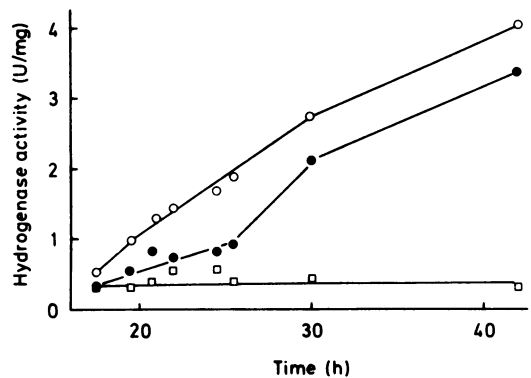

FIG. 4. Kinetics of hydrogenase activity in the presence of nickel chloride and chloramphenicol. Cells were grown as described in the legend to Fig. 1. The medium contained 10 μM EDTA. After 21 h of growth, 8 μM nickel chloride was added (●), and in a parallel culture, chloramphenicol was supplied in addition to nickel chloride (□). The control contained 8 μM nickel chloride from the beginning of the experiment (○).

TABLE 3. *Activities of key enzymes of autotrophic metabolism in crude extracts from cells grown in a nickel-deficient or nickel-supplemented medium*

Supplement added[a]		Enzyme activity (U/mg)[b]		
Nickel	EDTA	SH	PH	RuBPC
+	−	8.290	8.530	0.019
−	+	0.260	0.123	0.022
+	+	7.710	5.820	0.019
+[c]	+	7.840	2.580	0.036

[a] Cells were grown in a fructose-glycerol-containing minimal salts medium. Nickel chloride was added at a concentration of 0.8 μM. When 10 μM EDTA was present, 8 μM nickel chloride was added.

[b] Enzyme activities of soluble hydrogenase (SH) and ribulosebisphosphate carboxylase (RuBPC) were determined with crude extracts as described. The membrane-bound hydrogenase activity (PH) was determined with the particulate fraction as described.

[c] Nickel was added after 21 h of growth.

bisphosphate carboxylase was selected as an indicator enzyme for the carbon dioxide-fixing system. The data are summarized in Table 3. After 32 h of growth of the culture in fructose-glycerol medium with or without EDTA, and with or without nickel, both hydrogenases exhibited the same activity pattern. The formation of active membrane-bound hydrogenase was inhibited by EDTA, similar to the soluble hydrogenase. The addition of nickel could compensate for the inhibition by EDTA.

Ribulosebisphosphate carboxylase present at a relatively low specific activity under the growth conditions employed did not vary significantly with respect to nickel availability. The observation indicates that nickel is not essential for carbon dioxide fixation. This is supported by the fact that the utilization of formate did not require nickel as a divalent cation. As previously reported, formate is metabolized by *A. eutrophus* via the Calvin cycle (7). Thus, the results of Table 3 demonstrate that only the energy generation from hydrogen is nickel dependent.

DISCUSSION

The results presented in the preceding section clearly establish a specific function of nickel in the synthesis of active soluble and membrane-bound hydrogenases in *A. eutrophus*. Other divalent metal ions such as manganese, copper, zinc, and cobalt cannot substitute for nickel. Bartha and Ordal (2) failed to demonstrate a difference in hydrogen uptake between cells grown in a nickel-deficient and a nickel-supplemented medium. Presumably, traces of nickel were still present as a contaminant in the medium, particularly in the iron supplement even after purification of the nutrients by extraction

procedures. Our data indicate that the demand of *A. eutrophus* for nickel to form active hydrogenase is relatively low. Under autotrophic conditions cells grew equally well irrespective of whether nickel was supplied to the nonpurified medium, and no significant difference in soluble hydrogenase activity was observed. Thus, the use of chelating agents appeared to be the most efficient technique for rendering the traces of nickel ions inaccessible to the cells. Chelators have been successfully employed in demonstrating a specific nickel requirement for carbon monoxide dehydrogenase formation in *C. pasteurianum* (5). EDTA specifically has been reported to be a potent inhibitor of autotrophic growth of several hydrogen-oxidizing bacteria, since it prevented nickel ions from being taken up by the cells (15).

The heterotrophic system employed in this investigation was initially designed to provide a maximum level of hydrogenase activity under nonautotrophic conditions of growth. The regulatory mechanism that accounts for the dramatic increase of hydrogenase activity during the transition from fructose to glycerol is not yet known and is the subject of current investigations. In this study, the heterotrophic system provided a useful tool for elucidating the function of nickel in autotrophic metabolism. It enabled us to grow the cells in the presence of EDTA, since growth on fructose and glycerol was not inhibited by this chelator. We showed that soluble hydrogenase activity reached about 30% of the derepressed level when the cells were cultivated without the addition of nickel. Moreover, in the presence of EDTA, almost no hydrogenase activity was detected. However, the addition of nickel could compensate for the inhibition of active hydrogenase synthesis in the presence of EDTA. This revealed that under these conditions the cells were specifically lacking nickel and not iron for the synthesis of active hydrogenase. Iron was supplied in excess since it is an essential component of the enzyme which belongs to the iron-sulfur proteins (13). The fact that nickel did not stimulate hydrogenase activity when chloramphenicol was present suggests that the cation does not modulate the enzyme per se, by converting it from an inactive to an active state, but rather is required for the synthesis of active soluble and membrane-bound hydrogenases. The addition of nickel to a cell extract did not lead to a reactivation of soluble hydrogenase. Furthermore, purified enzyme does not depend on the presence of free nickel ions during the catalytic reaction (K. Schneider, personal communication).

The failure to reactivate hydrogenase in cell

extracts by the addition of nickel does not eliminate the possibility that nickel is part of the active enzyme. Molybdoenzymes such as nitrate reductase and formate dehydrogenase of *Escherichia coli* also cannot be converted to their active forms in cell extracts. However, in contrast to our results, reactivation occurs upon incubation of the cells with molybdate even in the presence of chloramphenicol (14). Nickel stimulates hydrogenase activity only when the cells are able to perform protein synthesis.

Recently, Diekert et al. (6) reported that in *Methanobacterium thermoautotrophicum* nickel is incorporated into a compound which is considered to be identical to F_{430}, a factor whose biological function is not yet known. It was suggested that the specific nickel requirement for the formation of carbon monoxide dehydrogenase in *C. pasteurianum* indicates a nickel complex as the prosthetic group of this enzyme (5). Furthermore, nickel has been identified as a component of jack bean urease (8).

Our data clearly restrict the role of nickel in autotrophic metabolism to energy conversion by hydrogen oxidation and show that carbon dioxide fixation does not depend on the availability of nickel. The results are too preliminary to draw a conclusion on the particular function of nickel in the synthesis of active hydrogenases. Nickel might be a constituent of (i) both hydrogenase proteins, (ii) a regulatory protein which is essential for the synthesis of the hydrogenases at the transcriptional or translational level, or (iii) a factor which is necessary to convert the hydrogenases into a catalytically active configuration similar to the ferredoxin-thioredoxin-modulated activation of chloroplast enzymes (4).

ACKNOWLEDGMENTS

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