Evidence for Nickel in the Soluble Hydrogenase from the Unicellular Green Alga *Scenedesmus obliquus*

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Z. Naturforsch. **49 c**, 33–38 (1994); received November 22, 1993

Scenedesmus obliquus, Hydrogenase, Nickel

Cultures of the green alga *Scenedesmus obliquus* were grown in the presence of either the chelating reagent EDTA or $NiCl_2$ in various concentrations and assayed for hydrogenase catalyzed photohydrogen evolution after an anaerobic dark adaptation period. Cultivation of algae in the presence of 100 μM EDTA inhibited the formation of hydrogenase activity by 37%. After a cultivation of the cells in the presence of 5–20 μM $NiCl_2$ photohydrogen evolution was increased by 20–40%. Addition of EDTA up to a final concentration of 1.5 mM had no effect on the activity of hydrogenase in cell-free hydrogenase preparations.

Cultures grown in the presence of radioactive $^{63}NiCl_2$ incorporated ^{63}Ni in a parallel fashion to the cell growth. In radioactive labeled hydrogenase preparations a co-elution of radioactivity and hydrogenase activity could be observed using gel filtration chromatography.

Introduction

Hydrogenases catalyze the reversible oxidation of molecular hydrogen as indicated by the following equation:

$$H_2 + 2\,e^-\text{-}(\text{acceptor}_{[ox]}) \rightleftharpoons 2\,H^+ + 2(e^-\text{-}\text{acceptor}^-_{[red]}).$$

The enzyme hydrogenase was first described by Stevenson and Stickland 1931. Today, hydrogenases are well known and characterized enzymes in various prokaryotic microorganisms (Przybyla *et al.,* 1992; Adams, 1990; Wu and Mandrand, 1993 for review). Since the fundamental investigations of Gaffron, who first discovered the ability of the unicellular green alga *Scenedesmus obliquus* to metabolize hydrogen under appropriate anaerobic conditions (Gaffron, 1939; Gaffron, 1940; Gaffron and Rubin, 1942), hydrogenases were found in various taxonomic classes of algae (Kessler and Maifahrt, 1960; Kessler, 1974; Kessler, 1978). Further research focussed on the physiological conditions and requirements necessary for light-dependent hydrogen metabolism in green algae and cyanobacteria (Bothe *et al.,* 1978; Kerfin and Böger, 1982; Almon and Böger, 1984; Kentemich *et al.,* 1990; Senger and Bishop, 1979; Randt and Senger, 1985).

Hydrogenases can be distinguished according to their cellular location or their *in vivo* electron donators or acceptors. Furthermore, most bacterial hydrogenases can be classified with respect to the metal composition of their reactive centers and are generally divided in Fe hydrogenases or FeNi(Se) hydrogenases (Przybyla *et al.,* 1992; Adams, 1990). Recently, a new type of hydrogenase lacking nickel ions as well as iron-sulfur clusters was found in methanogenic archaebacteria (Zirngibl *et al.,* 1992). Until now about 30 microbacterial hydrogenases have been characterized. In bacterial hydrogenases the absence of nickel seems to be more an exception than the rule.

In previous communications the effect of carbon monoxide and cyanide on hydrogenase activity of the eukaryotic alga *Scenedesmus* was investigated (Hartman and Krasna, 1964). Both, the anaerobic adaptation and the active hydrogenase, were shown to be inhibited by carbon monoxide and cyanide. In addition, the application of iron chelators was used to inhibit the formation of an active hydrogenase. Furthermore, it was shown that decreased concentrations of iron in culture media resulted in lower hydrogenase activity in *Scenedesmus* (Kessler, 1968; Yanagi and Sasa, 1966).

Abbreviations: EDTA, ethylenediaminetetraacetic acid; PCV, packed cell volume.

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Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/94/0100–0033 $ 01.30/0

From these studies it was concluded that most probably iron is part of the active site of the hydrogenase from *Scenedesmus.*

However, until now there is no information available whether hydrogenases of eukaryotic algae contain nickel or not. In the present paper we study the influence of nickel during cell growth on the formation of hydrogenase activity in *Scenedesmus.*

Experimental

Organism and growth conditions

Cells of the wild type of the green alga *Scenedesmus obliquus* strain D_3 (Gaffron, 1939) were grown autotrophically as described by Senger and Bishop (1972). Cultures were aerated with 3% CO_2 in air and illuminated with fluorescent white light (20 W/m^2).

Determination of hydrogen and oxygen

Hydrogen (H_2 evolution assay; *in vivo*) and oxygen evolution were measured polarographically using a Micro-Clark electrode (Gilson Medical Electronics, Middleton, Wisconsin, U.S.A.) at 28 °C under saturating incandescent light. The electrode was calibrated for H_2 measurements with a gas mixture containing 2% H_2 (Messer, Griesheim) or for O_2 measurements with oxygen saturated buffer, respectively. The solubility of H_2 and O_2 in aqueous solutions was calculated according to data from the literature (May, 1967).

For determination of hydrogen uptake in cell-free homogenates methylviologen (10 mM) was used as electron acceptor (H_2 evolution assay; *in vitro*).

For measurements of hydrogen production in cell-free preparations dithionite reduced methylviologen (10 mM) was used as electron donor. Reaction was started by the addition of 0.1 ml of the sample (H_2 uptake assay; *in vitro*).

Determination of basic parameters

Packed cell volume (PCV) was determined by centrifugation of aliquots of the cell suspension in hematocrit tubes for 5 min at 1400 × *g*.

Chlorophyll content was determined after extraction with hot methanol using the formula given by Porra (1991).

Protein was quantified by the method of Bradford (1979).

Hydrogenase activation

Cultures were harvested by centrifugation and washed twice with bis-Tris/HCl buffer (50 mM, pH 7.5). Cells were adjusted to a PCV of 20 µl/ml (for *in vivo* measurements) or 500 µl/ml for preparing cell-free homogenates and placed in 50 ml flasks sealed with serum stoppers. The samples were repeatedly vacuum degassed, flushed with nitrogen and finally shaken for the indicated times at 30 °C in darkness.

Cell-free preparation

Anaerobically adapted cells were disrupted with a Vibrogen cell mill (Bühler, Tübingen) as described by Senger and Mell (1977).

Incorporation of ^{63}Nickel

Incorporation of radioactive ^{63}Ni was determined by liquid scintillation counting using a Betamatic liquid scintillation counter (Kontron, München). For the determination of ^{63}Ni in whole cells, aliquots of the culture were centrifuged and washed twice with bis-Tris/HCl buffer (50 mM, pH 7.5).

Materials

All chemicals were of analytical reagent grade and purchased from Merck, Darmstadt; $^{63}NiCl_2$ was obtained from Amersham, Braunschweig.

Results and Discussion

Effect of nickel on hydrogenase activity

Metal chelating reagents, especially EDTA, were previously reported to be effective in inhibiting the formation of hydrogenase activities in several bacteria (Pedrosa and Yates, 1983; Papen *et al.,* 1986; Friedrich *et al.,* 1981). In these studies the inhibiting effect could be prevented specifically by the addition of nickel salts. Therefore, it was concluded that EDTA inhibits the uptake of nickel by those organisms.

In the present communication we investigate the effect of EDTA and nickel ions on hydrogenase activity in the eukaryotic green alga *Scenedesmus obliquus.*

For this purpose, cultures were grown for 9 d in the presence of EDTA. During this time aliquots of the culture were transferred to new media containing the same concentration of EDTA every 3 d. This was done to deplete the internal amount of nickel ions in the algae. The chosen time interval (3 d) corresponds to the normal growth phase of the algae. Because no hydrogenase activity could be detected under aerobic growth conditions, algae were harvested, followed by a dark incubation under anaerobic N_2 atmosphere for 3 h, and subsequently assayed for hydrogenase mediated photohydrogen evolution (*in vivo* hydrogenase evolution assay). After 3 h of anaerobic adaptation maximum rates of hydrogenase mediated photohydrogen evolution were obtained and the rates remained constant (Fig. 1). For all experiments an adaptation period of 3 h has been used.

Increasing concentrations of EDTA in the culture media resulted in decreased hydrogenase activities (Fig. 2). A final concentration of 100 μM EDTA caused most effective suppression of hydrogenase activity (37%). For investigation of the effect of nickel ions on hydrogenase activity, cultures were grown in the presence of $NiCl_2$ (5–20 μM final concentration) for 3 d and subsequently assayed for photohydrogen evolution. The addition of various amounts of $NiCl_2$ to the culture medium resulted in a stimulation of hydrogenase activity in these cultures. After the addition of nickel chloride to final concentrations from 5 μM to 20 μM hydrogenase activity was increased up to 50% (Fig. 2).

It has to be mentioned that our standard culture medium is not supplemented by nickel salts. Thus, nickel ions can only be present as contaminants of trace elements in the media. The concentration of nickel deriving from impurities of the chemicals was estimated to about 50 nM. That it was below 85 nM was demonstrated by atomic absorption spectroscopy (data not shown). The former stimulation of hydrogenase activity by $NiCl_2$ indicates that the availability of nickel in our standard culture media might be limited but not insufficient during cell growth with respect to subsequent formation of hydrogenase activity. This could be an explanation for the rather moderate repression effect of EDTA in the culture experiments, since more drastic repression effects have been reported for bacterial hydrogenases.

For our *in vivo* experiments such concentrations of EDTA and $NiCl_2$ were chosen which ensure significant repression effect on the forma-

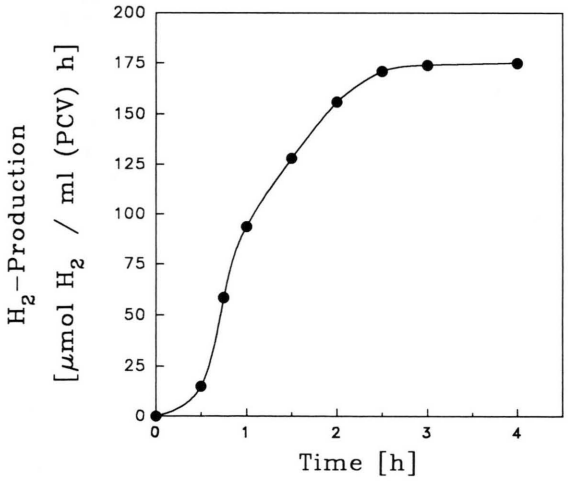

Fig. 1. Photohydrogen evolution during the anaerobic adaptation of *Scenedesmus* cells. Cultures were grown autotrophically for 3 d. Algae were harvested and adapted to aerobic conditions by degassing and flushing with nitrogen. The photohydrogen evolution (—●—) was measured by transferring aliquots of the algal suspensions containing 40 μl PCV in 2 ml buffer into the reaction chamber of the electrode. Photohydrogen evolution was measured by illumination with saturating incandescent light.

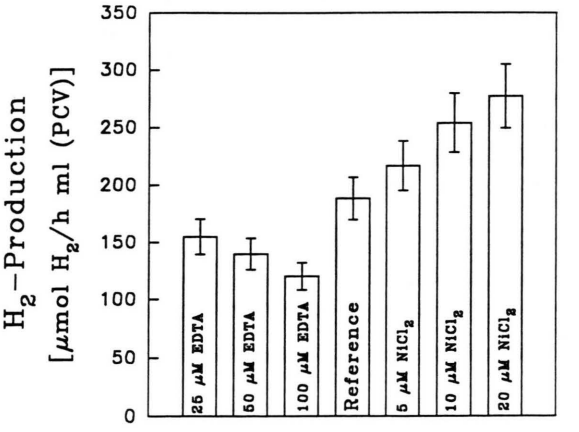

Fig. 2. Influence of EDTA or $NiCl_2$ on photohydrogen evolution of *Scenedesmus in vivo*. Cultures were grown in the indicated concentrations of EDTA or $NiCl_2$. After autotrophic cultivation cells were harvested, anaerobically adapted, and assayed for hydrogenase activity by determination of photohydrogen evolution. Data are averages of three independent experiments. Concentrations of EDTA and $NiCl_2$ are as indicated in the figure.

tion of hydrogenase activity without affecting cell growth. Since the photosynthetic electron transport pathway is required for the hydrogenase-catalyzed photohydrogen evolution, we also checked photosynthetic oxygen evolution when algae were grown in the presence of EDTA or $NiCl_2$. Furthermore, cell growth was monitored by determination of packed cell volume as well as by determination of the chlorophyll contents. For this purpose, cultures were grown in the presence of the highest concentration of EDTA or $NiCl_2$ being used in the experiments described above. Cultures grown in the presence of 100 μM EDTA or 20 μM $NiCl_2$, respectively, showed the same packed cell volume, oxygen evolution and chlorophyll content as the control samples.

We also investigated the effect of EDTA and $NiCl_2$ after anaerobic adaptation on the already activated hydrogenase *in vitro*. The hydrogenase activity was then determined after preparing cell-free suspensions of anaerobically adapted algae which were grown in standard media without supplements. Enzyme activity was monitored by hydrogenase-catalyzed oxidation of reduced methylviologen (*in vitro* hydrogen evolution assay). EDTA was added up to a final concentration of 1.5 mM. $NiCl_2$ was also added up to a final concentration of 1 mM. Both supplements had no effect on the already present hydrogenase.

It has been also reported for *Azotobacter chroococcum* (Pedrosa and Yates, 1983) that there is no inhibition effect of those reagents on the already preformed hydrogenase, even if the concentrations of the used chelators were up to ten times higher than the concentrations causing the repression of hydrogenase activity in growing cells. Similar observations are reported for *Alcaligenes eutrophus* (Friedrich *et al.*, 1981).

This fact demonstrates that the availability of nickel in the culture medium is an important factor for the formation of an active hydrogenase.

Incorporation of ^{63}Ni into growing cells of Scenedesmus and in partial purified hydrogenase preparations

For investigation of a possible incorporation of nickel ions during cell growth *Scenedesmus* cells were grown autotrophically in the presence of 3 μM $^{63}NiCl_2$ (specific radioactivity 2 μCi/ml; 74 kBq/ml; 4.5×10^6 cpm/ml) for 4 d. At the times indicated, aliquots of the culture were removed, centrifuged and washed twice with buffer. Since there was no significant radioactivity in the buffer after the final wash, the determinated radioactivity was attributed to nickel incorporated into the cells. An increasing incorporation of radioactivity in parallel to the cell growth (Fig. 3) was observed. Within 60 h cells have reached their early stationary phase and no additional radioactivity was incorporated. The maximum amount of incorporated radioactivity was 8×10^4 cpm/ml PCV.

For further clarification whether the supplemented nickel is incorporated in the hydrogenase, a culture grown in the presence of $^{63}NiCl_2$ was adapted to anaerobic conditions and the cells were subsequently disrupted. The crude homogenate was centrifuged at 300,000 × *g* for 1 h at 4 °C. 300 μl of the supernatant containing both, hydrogenase activity and radioactivity, was applied to a Sephadex G-200 column (1.0 × 38 cm). Fractions of 1 ml were collected (flow rate 11 ml/h) and assayed for incorporated radioactive nickel and hydrogenase activity. For a qualitative determination of hydrogenase activity we used the H_2-dependent reduction of methylviologen. (The soluble hydrogenase catalyzes both methylviologen reduction with molecular hydrogen and H_2 evolution from reduced methylviologen *in vitro*.) Both co-eluted at

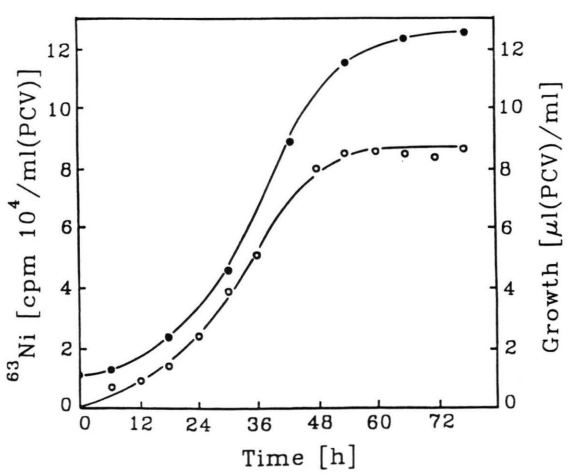

Fig. 3. Incorporation of radioactive $^{63}NiCl_2$ by *Scenedesmus* cells. Cultures were grown in the presence of $^{63}NiCl_2$ (2 μCi/ml). At the times indicated aliquots of the culture were removed for determination of PCV (—●—) and incorporated ^{63}Ni (—○—).

17–22 ml. The maximum radioactivity eluted at 21 ml (Fig. 4). Hydrogenase activity was detected in fractions 12 to 21, but the maximum hydrogenase activity was shown to elute in fraction 19 to 21. As described earlier, hydrogenase tends to form higher molecular weight aggregates with decreased enzyme activity (Schnackenberg *et al.*, 1993). The hydrogenase activity in the fractions 12–19 might be caused by those hydrogenase aggregates.

From our culture experiments we conclude that nickel ions are involved in the formation of hydrogenase activity in *Scenedesmus*. The incorporation of nickel during cell growth as well as the co-elution of radioactive nickel and hydrogenase activity provides further evidence for the presence of nickel in the hydrogenase from *Scenedesmus*. Since there was no effect of EDTA or $NiCl_2$ on the already activated hydrogenase as shown by *in vitro* experiments, we conclude that the incorporation of nickel in the hydrogenase enzyme occurs during cell growth as demonstrated by the labeling experiments using radioactive nickel. Therefore, we assume that nickel ions are inserted into the reactive center of the hydrogenase of *Scenedesmus obliquus* during its growth. In further studies the location of nickel, the molar amount of nickel in the hydrogenase enzyme or its subunits as well as the function of nickel will be investigated.

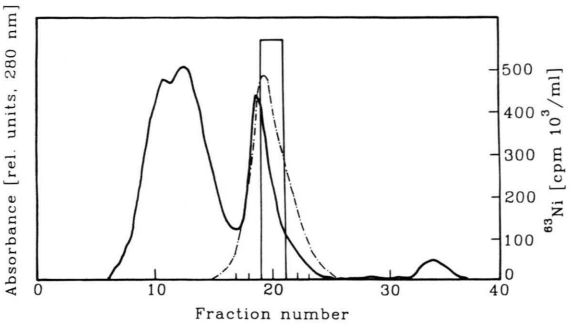

Fig. 4. Gel filtration chromatography of a ^{63}Ni-labeled hydrogenase preparation. Cultures grown in the presence of $^{63}NiCl_2$ were harvested, adjusted to a PCV of 500 µl/ml in 50 mM bis-Tris/HCl buffer (pH 7.5) and anaerobically adapted. After disruption and centrifugation (300,000 × *g*, 4 °C, 1 h) 300 µl of the supernatant, containing all hydrogenase activity, were applied to a Sephadex G 200 column (38 × 1 cm). Fractions of 1 ml were collected (flow rate 11 ml/h) and assayed for hydrogenase activity (methylviologen reduction, *in vitro* H_2 uptake assay) and radioactivity. (——) Absorbance, $OD_{280\,nm}$; (–·–·–) incorporated radioactivity; open bar: main hydrogenase activity, arbitrary units.

Acknowledgements

This work was supported by the Bundesministerium für Forschung und Technologie (Biologische Wasserstoffgewinnung).

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