Enhancement of hydrogenase activity in \textit{Rhodopseudomonas capsulata} by nickel

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1. INTRODUCTION

Photoautotrophic growth on H$_2$, CO$_2$ and (NH$_4$)$_2$SO$_4$ was clearly demonstrated for the purple photosynthetic bacteria in 1962 by Ormerod and Gest [1]. Even earlier, the enzyme responsible for H$_2$ uptake had been assayed in cell-free extracts of these bacteria, shown to be membrane-bound and not to require a chelatable metal ion for activity [2]. Also this enzyme, hydrogenase, is believed to be essential for chemioautotrophic growth (aerobically in darkness) of \textit{Rhodopseudomonas capsulata} [3]. The membrane-bound enzyme has now been solubilized from \textit{R. capsulata} [4] and purified to homogeneity from \textit{Rhodospirillum rubrum} [5].

In these organisms, hydrogenase operates essentially unidirectionally to take up H$_2$ during photo-reduction [6,7]; the observed H$_2$ production is mediated by another enzyme system, nitrogenase [7,8]. Recently, an additional function has been ascribed to hydrogenase, the recycling of H$_2$ produced during nitrogen fixation [9].

To characterize mutants of \textit{R. capsulata} lacking the capacity to grow photoautotrophically because of an inability to take up H$_2$ and to demonstrate the effect of this loss on nitrogen fixation, it was necessary to understand the factors influencing the production of hydrogenase. H$_3$ is one factor that has been shown to stimulate hydrogenase formation [10], while O$_2$ has been implicated in inactivation or repression [11]. In addition, nickel has been shown to activate and stabilize the soluble hydrogen dehydrogenase of the Gram-positive organism \textit{Nocardia opaca} 1b in vitro [12]. Following a recent report that nickel was required for active soluble and membrane bound hydrogenase formation in \textit{Alcaligenes eutrophus} [13], the effect of this transition metal on hydrogenase activity in \textit{R. capsulata} was examined. Evidence is presented which implicates nickel in the production of active hydrogenase in this photosynthetic bacterium.

2. MATERIALS AND METHODS

2.1. Bacterial strain

\textit{R. capsulata} B100 is a derivative of wild-type B10 (described in [14]) spontaneously cured of bacteriophage [15].

2.2. Media and growth conditions

The minimal medium used, designated RCV, contained 30 mM DL-malate, 7.5 mM (NH$_4$)$_2$SO$_4$ and constituents as specified for RCVB in Johanson and Gest [16], except that thiamine-hydrochloride, the only vitamin supplied, was added at 1 mg/liter. For growth with glutamate as nitrogen source, RCV was modified by omission of ammonium salts and addition of 7.0 mM L-glutamate.
All cultures were grown photosynthetically under anaerobic conditions in completely filled screw-capped test tubes or bottles, incubated in a glass-sided water bath at 35°C and illuminated with a bank of three 60-W Lumiline incandescent lamps (approx. 8000 lux).

2.3. Preparation of cells and extracts

Cells were harvested by centrifugation at 27000 × g for 30 min at 15°C, washed twice in 20 mM Tris-HCl buffer (pH 8.0) and resuspended in 1/5 vol. of the same buffer. The concentrated cells were flushed with argon for 10–15 min and used for whole-cell assays. For the preparation of extracts, cells were similarly harvested except 100 mM Tris-HCl buffer, pH 8.0, was used. Subsequent fractionations were made under H₂ except where indicated. The cell concentrate was passed through a French press at 16000 psi, 23°C, the extract centrifuged at 27000 × g for 30 min, 15°C, and the supernatant used as the cell-free extract. The cell-free extract was recentrifuged (180000 × g for 2 h at 15°C) and the resultant sediment was resuspended and homogenized aerobically in 20 mM Tris–HCl, pH 8.0, to a protein concentration of 3–6 mg/ml. This particulate fraction was then flushed with H₂ before assay or storage at -20°C.

2.4. Hydrogenase assay

Hydrogenase activity of whole cells or extracts was assayed as H₂ dependent reduction of methylene blue (MB) essentially as in [4]. 1 ml of H₂ saturated 0.12 mM MB in 20 mM Tris–HCl, pH 8.0, was injected into a disposable anaerobic cuvette previously gassed with H₂. Then 25–100 μl of H₂ saturated cell suspension or extract (containing 0.1–0.4 mg protein) was added. MB reduction was followed at room temperature, spectrophotometrically at 570 nm (εₘₘₙ = 16.5/mM/cm; Takakuwa, unpublished; see also [4]). Controls with argon replacing H₂ were included to evaluate non-specific reduction. The addition of 1% Triton X-100 had no stimulatory effect on the assay in whole cells [4].

2.5. Other determinations

Protein in whole cells, crude extracts and particulate fractions was determined by the Lowry method [17] after digestion of the sample with 0.2 M NaOH for 1 min at 100°C. Bovine serum albumin (Sigma) was used as the standard. Absorbances were determined with a Coleman Junior II Spectrophotometer at 660 nm.

2.6. Chemicals

Methylene blue, crystalline NiCl₂·6 H₂O and analytical grade ZnSO₄·7 H₂O were purchased from Sigma Chemical Company. Crystalline CoCl₂·6 H₂O and analytical grade CuSO₄ were from Matheson Coleman and Bell and Mallinckrodt Chemical Works, respectively. Acetohydroxamic acid was generously prepared by Thomas Mawhinney and was >99.1% pure. All other chemicals were analytical grade.

3. RESULTS

Hydrogenase activity in R. capsulata cultures is present in cells grown in a variety of growth modes and appears to be stimulated when H₂ is present during growth [10]; for example, during growth in a medium allowing nitrogenase derepression and consequent H₂ evolution. When 1 μM NiCl₂·6 H₂O was added to cells grown without exposure to H₂ or with nitrogenase depression, the hydrogenase activity of whole cells was increased 3–8-fold as seen in Table 1. Although there was often considerable variation in the specific activity obtained, there was always at least a 2-fold stimulation when cells were grown with added nickel.

To determine the optimal concentrations of Ni²⁺ for the stimulation of hydrogenase activity in R. capsulata B100, cultures were grown with different amounts of added Ni²⁺, the cells harvested and the enzyme assayed. During this determination it was found that growth rates and cell yields were not detectably affected by the addition of up to 100 μM NiCl₂·6 H₂O to the growth medium. Although no attempt was made to eliminate trace Ni²⁺ from the medium, it can
Table 1

Effect of nickel on hydrogenase activity in *Rhodopseudomonas capsulata* B100 whole cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Ni²⁺ addition</th>
<th>Spec. act. (µmol MB reduced/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate + (NH₄)₂SO₄</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>Malate + (NH₄)₂SO₄</td>
<td>+ d</td>
<td>16.2</td>
</tr>
<tr>
<td>Malate + glutamate</td>
<td>-</td>
<td>11.3</td>
</tr>
<tr>
<td>Malate + glutamate</td>
<td>+</td>
<td>32.5</td>
</tr>
</tbody>
</table>

a Ni²⁺ was added to the culture medium and cells were grown photosynthetically for 24 h at 35°C before harvesting and assaying hydrogenase in whole cells. Results shown are typical of three independent experiments and are the average of duplicate assays.

b Malate + (NH₄)₂SO₄ = RCV medium.

c Malate + glutamate = RCV lacking (NH₄)₂SO₄ supplemented with 7.0 mM l-glutamate.

d Ni²⁺ added to 1 µM final concentrations as NiCl₂·6 H₂O.

be seen in Fig. 1 that the addition of 1–10 µM Ni²⁺ resulted in maximum enzyme activity under these conditions.

The metal specificity for this enhancement of hydrogenase activity is shown in Table 2. It should be pointed out that the minimal medium contained as "trace elements" 45 µM H₃BO₃, 0.17 µM Cu(NO₃)₂·3 H₂O, 9.4 µM MnSO₄·H₂O, 3.1 µM Na₃MoO₄·2 H₂O and 0.83 µM ZnSO₄·7 H₂O. Additional supplements of Zn²⁺ or Cu²⁺ did not greatly increase the hydrogenase activity of whole cells. To the contrary, 10 µM Cu²⁺, which was the only metal supplement to inhibit growth, consequently abolished hydrogenase activity. At 10 µM, Co²⁺ gave a 2-fold as compared to a 5-fold increase in activity for Ni²⁺ at the same concentration.

Experiments in medium lacking trace elements (data not shown) corroborated that Co²⁺, Cu²⁺ and Zn²⁺ (and additionally manganese and molybdenum) replaced Ni²⁺ in its effect on hydrogenase activity only poorly or not at all.

The effect of Ni²⁺ on the activity of hydrogenase in cell-free extracts and particulate fractions was investigated. The higher specific activities seen in whole cells as a result of Ni²⁺ supplementation were maintained in extracts. However,

![Table 2](https://academic.oup.com/femsle/article-abstract/12/4/359/571906)
no stimulation was obtained with addition of Ni\(^{2+}\) in vitro regardless of the previous culture condition of the cells. In addition, no increase in activity was obtained when the particulate fraction was dialyzed against 10 \(\mu\)M Ni\(^{2+}\) overnight before assay. Finally, in confirmation of earlier findings \([2,5,18]\), little inhibition (25% or less) of activity was found after the addition of the chelators, EDTA, citrate, or acetohydroxamic acid, to the particulate fraction in the presence of deoxycholate (data not shown).

4. DISCUSSION

Nickel was shown to be specifically required for autotrophic growth of *Hydrogenomonas* in 1965 by Bartha and Ordal \([19]\). Since then, this transition metal has been demonstrated to be involved in a number of metabolic processes \([20,21]\) and to be a component of jack bean urease \([22]\) and carbon monoxide dehydrogenase from *Clostridium thermoceticum* \([23]\). Experiments with autotrophic microorganisms have demonstrated that the effect of nickel is specific to hydrogen utilization \([12,13]\).

In this study, cells of *R. capsulata* were found to have an increased activity of hydrogenase when the culture medium was supplemented with small quantities of Ni\(^{2+}\) (1–10 \(\mu\)M). The effect of Ni\(^{2+}\) appeared to be specific; Cu\(^{2+}\) and Zn\(^{2+}\) were completely ineffective, while Co\(^{2+}\) was only partially able to substitute for nickel. Addition of Ni\(^{2+}\) to extracts of cells grown in Ni\(^{2+}\) limiting medium showed no increase in hydrogenase activity. Finally, it was shown that the particulate enzyme was not inhibited by chelators. Although the presence of nickel in hydrogenases purified from photosynthetic bacteria has not been investigated, it is known that all these enzymes contain Fe \([5]\), presumably as an essential component of the active site. Therefore, the lack of inhibition, even of purified *R. rubrum* hydrogenase \([5]\), by chelators \([2,18]\), suggests that the metal or metals are tightly bound or are buried within the protein.

There are several possibilities to explain these observations: (a) Nickel metabolism interacts with iron metabolism and stimulates production of iron containing enzymes \([24]\). (b) Nickel is an essential component of the enzyme and must be present during synthesis to be incorporated into the nascent polypeptide \([13,23]\). (c) The biosynthesis of the enzyme is nickel-dependent \([20]\). (d) Activation factors necessary for hydrogenase activity are dependent on nickel for synthesis or function \([13]\). (e) An essential electron transport component associated with hydrogenase (and measured in the assay used here) is dependent on nickel for synthesis or function. The first possibility is unlikely since, as demonstrated by assays of nitrogenase, there was not a general increase in the activities of iron-rich proteins when the cells were grown in the presence of Ni\(^{2+}\). In case (b), as opposed to the latter three, the purified enzyme would contain nickel. Therefore, a definitive explanation for the enhancement of hydrogenase activity in *R. capsulata* must await the purification of the enzyme.

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REFERENCES