Phytochemistry, Vol 26, No 3, pp 637–640, 1987
Printed in Great Britain

0031-9422/87 $3.00 + 0.00
Pergamon Journals Ltd

HYDROGENASE FROM THE UNICELLULAR CYANOBACTERIUM, *MICROCYSTIS AERUGINOSA*

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(*Received* 1 *July* 1986)

Key Word Index—*Microcystis aeruginosa*, cyanobacterium, hydrogenase, purification, properties

Abstract—A hydrogenase was isolated from a unicellular and non-nitrogen-fixing cyanobacterium, *Microcystis aeruginosa* strain NIES 44. The enzyme was easily solubilized and was capable of evolving hydrogen gas in the presence of reduced methyl viologen and benzyl viologen. The enzyme was stimulated by divalent ions and showed a pH optimum around 6.8. The M_r of the enzyme, estimated by gel filtration, was 50000.

INTRODUCTION

Hydrogenases have been extensively studied in physiological investigations of nitrogen-fixing cyanobacteria [1–4]. One interpretation of the role of hydrogenase is in the recovery of hydrogen gas which is evolved by an irreversible side reaction of nitrogenase. Besides this 'uptake' hydrogenase activity, it has been suggested that a 'reversible' hydrogenase may catalyse hydrogen evolution, not only in nitrogen-fixing [5–8], but also non-nitrogen-fixing cultures and species [9–11].

Hydrogenases have been partially purified from the following cultures of filamentous species: nitrogen-fixing *Anabaena cylindrica* [12], nitrogen-fixing *Anabaena* sp. strain 7120 [13], natural water bloom of *Oscillatoria limnetica* [14], which can metabolize molecular hydrogen during sulphide-dependent anoxygenic photosynthesis [9], natural water bloom of *Spirulina maxima* [15] and natural water bloom of *Mastigocladus laminosus* [16]. In marked contrast to the filamentous cyanobacteria, there is little information available about hydrogenase in unicellular and non-nitrogen-fixing cyanobacteria, in particularly those with a hydrogen evolving capability. It has been reported that the axenic culture of a unicellular and non-nitrogen-fixing *Microcystis aeruginosa* strain NIES 44 [17] and other species of *Microcystis* [18] can evolve molecular hydrogen endogenously in dark and anaerobic conditions and consume it by oxyhydrogen and anaerobic light-dependent reactions. These considerations have prompted us to study some properties of the hydrogenase in *M. aeruginosa* and to compare these properties with those observed for the enzymes from other cyanobacteria.

RESULTS AND DISCUSSION

When intact cells of *M. aeruginosa* were broken by a French press, most of the hydrogenase activity was recovered in the supernatant fraction (Table 1). The supernatant fraction evolved hydrogen in the presence of reduced methyl viologen and consumed hydrogen in the presence of vitamin K_3, suggesting the occurrence of a 'reversible' hydrogenase. In *Anabaena* sp. strain 7120 [13], *Anabaena cylindrica* [12], *Spirulina maxima* [15] and *Mastigocladus laminosus* [16], the 'reversible' hydrogenase is known to be easily solubilized by gentle cell disruption procedures. Furthermore, two forms of 'reversible' hydrogenase, differing in kinetic and chromatographic properties and mediator specificity, have been isolated from *Oscillatoria limnetica* [14]. In the present study, a single band staining for hydrogenase was observed when a sample of the supernatant fraction was subjected to polyacrylamide gel electrophoresis (data not shown). In addition, DEAE-cellulose and Sephacryl S-200 chromatography failed to resolve the hydrogenase into more than one peak of activity (see below).

Table 1. Distribution of hydrogenase activity

Fraction	μl H_2 hr ml extract: H_2 evolution from 1 mM MV_{red}	H_2 uptake with 1 mM vitamin K_3
110000 *g* supernatant	141.5	2.32
110000 *g* pellet	7.1	0.13

Intact cells were broken by passing through a French press (800 kg/cm^2). The extract was centrifuged at 2400 *g* for 10 min and the supernatant from this step was centrifuged at 110000 *g* for 1 hr. MV was reduced by 5 mM $Na_2S_2O_4$.

When intact cells of *M. aeruginosa* were broken by sonication, the hydrogenase was also recovered in the soluble fraction. To further purify the enzyme, the solubilized hydrogenase was passed through DEAE-cellulose and Sephacryl S-200 columns. Table 2 summarizes the protein distribution, specific activities and the recovery of activity units in various steps of purification. On DEAE-cellulose chromatography, considerable amounts of the blue phycobiliproteins were removed at 0.1 M NaCl and the enzyme was eluted at 0.2 M NaCl (Fig. 1). However, the specific activity following this step was lower than that attained for the enzyme applied to the column. A crude fraction of ferredoxin was also recovered in the 0.3 M NaCl eluant. On Sephacryl S-200 chromatography, more blue pigments were separated from the hydrogenase, but the eluted enzyme exhibited a further decline in activity. This finding prevented attempts to further purify the enzyme. Consequently all characterizations were conducted with the partially purified enzyme that eluted from the Sephacryl column.

There were indications throughout the purification that the enzyme might be unstable when exposed to air. First, inclusion of reducing reagents such as DTT, cysteine-HCl and reduced glutathione in the enzyme buffer throughout the purification improved the yield of the enzyme. Secondly, if the solubilized enzyme was stored at 5° in air with DTT, half of the activity was retained after 5 hr. Thirdly, if the partially purified enzyme was frozen at −80° with DTT, half of the activity was recovered after 3 months. These findings are very similar to those reported for the partially purified preparations of 'reversible' hydrogenases from *S. maxima* [15] and *M. laminosus* [16]. In contrast, other studies have indicated that the 'reversible' hydrogenases from *Anabaena* 7120 [13] and *O. limnetica* [14] are quite resistant to inactivation by oxygen.

The M_r of the *M. aeruginosa* hydrogenase, determined by gel filtration on a calibrated Sephadex G-100 column, was estimated to be 50000. This value is smaller than those reported for the 'reversible' hydrogenases from *S. maxima* [15] and *Anabaena* 7120 [13]. The M_r of the *S. maxima* enzyme, determined on a calibrated Sephacryl S-200 column, was estimated to be 55000. In *Anabaena* 7120, results indicated that aggregation of the enzyme occurred in solution. The enzyme was first heated to 70° for 1 hr, three peaks of M_r 113000, 165000 and 55000 appeared on a calibrated G-200 column. The last protein species was obtained only with heat treatment.

The pH activity profile for the *M. aeruginosa* hydrogenase has a sharp pH optimum at pH 6.8–7.0 and is similar to that observed for the enzyme purified from *Anabaena* 7120 [13] and *O. limnetica* [14].

Table 2. Purification of hydrogenase

Step	Total protein (mg)	Total activity (unit*)	Specific activity (unit/mg)	Yield (%)
2400 *g* supernatant	665	495	0.745	100
110000 *g* supernatant	468	362	0.773	73.6
DEAE-cellulose	37.4	14.6	0.591	2.95
Sephacryl S-200	8.49	1.92	0.226	0.388

*1 unit = 1 μmole of H_2 evolved/hr

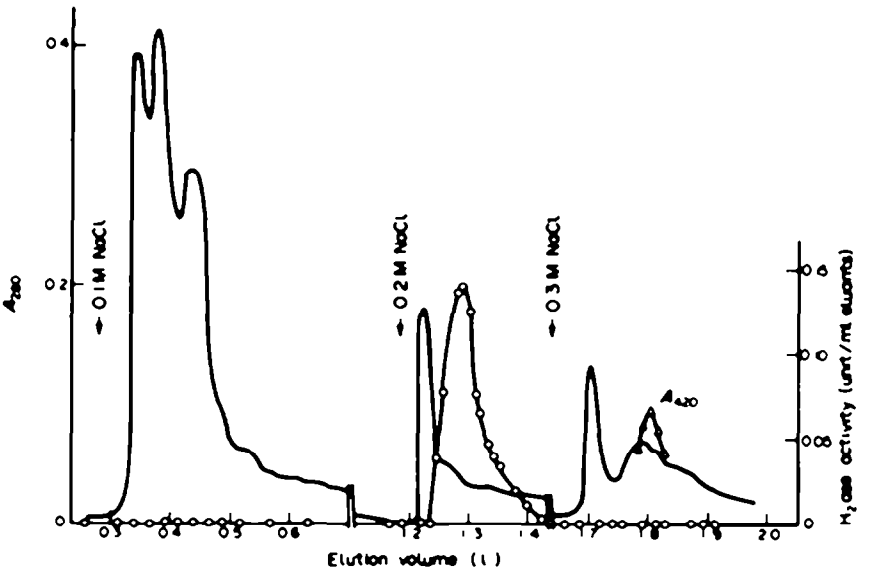

Fig. 1. DE 52 column chromatography of hydrogenase from *Microcystis aeruginosa*. — A_{280}. ⋯ A_{420}. ○ hydrogenase activity. A_{420} was measured to detect ferredoxin.

Like the *S. maxima* enzyme [15], the *M. aeruginosa* hydrogenase was stimulated by divalent cations except Cd^{2+} and Cu^{2+} (Table 3). The *M. aeruginosa* enzyme was stimulated (in order of increasing effectiveness) by Sn^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}. Although Zn^{2+} was the most effective cation with the *M. aeruginosa* enzyme, it did not stimulate the *S. maxima* enzyme. In addition, ATP and Mg^{2+} were found to inhibit the *S. maxima* enzyme but not that of *M. aeruginosa*.

The artificial electron carriers, methyl viologen and benzyl viologen, were the only effective electron donors. The ferredoxin fraction (Fig. 1) was not effective at the concentration tested. This result resembles those for *A. cylindrica* [12] and *O. limnetica* [14]. In the present study, the K_m value for methyl viologen was *ca* 0.51 mM in the hydrogen evolution assay. This value is the highest reported so far for 'reversible' hydrogenases. Values of 0.26 and 0.023 mM, 0.16 and 0.053 mM have been reported for *O. limnetica* [14], *S. maxima* [15] and *A. cylindrica* [12], respectively.

Our results have confirmed the occurrence of a soluble hydrogenase in *M. aeruginosa*, a unicellular and non-nitrogen-fixing cyanobacterium. The properties described here suggest that the enzyme may be a 'reversible' hydrogenase similar to that found in a number of the filamentous and nitrogen-fixing cyanobacteria. The physiological role of the 'reversible' hydrogenase is still obscure. It has been speculated that the enzyme plays a dominant role in an anaerobic environment [4]. We have previously demonstrated that, in dark and anaerobic conditions, intact cells of *M. aeruginosa* [17] and *S. platensis* [19] could evolve molecular hydrogen. It is not unreasonable to assume that the present enzyme is involved in this hydrogen evolution. However, further study is necessary to verify this suggestion. Further study is also necessary to reveal whether the hydrogen uptake activity in intact cells of *M. aeruginosa* is due to the present enzyme or another membrane-bound enzyme. A soluble 'reversible' hydrogenase together with a membrane-bound 'uptake' hydrogenase have been prepared from the French press extract of *Anabaena* 7120 [13]. In contrast to the 'reversible' hydrogenase, the 'uptake' hydrogenase cannot evolve hydrogen from reduced methyl viologen at an appreciable rate but it can consume hydrogen in the presence of electron acceptors. We have measured hydrogen uptake activity in our French press extract and found very little activity in the soluble and insoluble fractions when vitamin K_3 was used as a mediator (Table 1). This does not eliminate the possibility of the existence of an 'uptake' hydrogenase in *M. aeruginosa*, in view of the findings related to the *Anabaena* 7120 enzyme. The latter was found to be more labile than the 'reversible' hydrogenase and to react only with mediators having a positive midpoint potential. Currently we are investigating the conditions necessary for the reconstitution of hydrogen uptake activity in *M. aeruginosa* extracts.

Table 3. Effect of metal ions on hydrogenase activity

Metal ions	Relative activity of hydrogenase (%)
None	100
Mg^{2+}	156
Ca^{2+}	145
Mn^{2+}	159
Fe^{2+}	169
Co^{2+}	170
Ni^{2+}	184
Cu^{2+}	12.4
Zn^{2+}	199
Cd^{2+}	10.4
Sn^{2+}	112
Ba^{2+}	135

All metal ions were added at 1 mM. The activity of 100% was equivalent to 0.113 μl H_2/hr/ml of reaction mixture. The concentration of the partially purified hydrogenase was equivalent to 56.7 μg protein/2.24 ml of reaction mixture.

EXPERIMENTAL

An axenic strain of *M. aeruginosa* forma *aeruginosa* Kutzing NIES 44 [20, 21] was cultured in a modified MA medium [18] at 27–28° under periodic illumination (16 hr on/8 hr off) of 30–40 W/m² from cool white fluorescent tubes. The culture was bubbled with air during the light period. After 8–12 days of growth, the culture broth was gently sonicated using an ultrasonic washer to remove gas vacuoles and centrifuged at 2400 *g*. The pellet was washed with 50 mM Tris–HCl buffer, pH 7.5. The yield of the cyanobacterial cells was 10–20 g (wet wt) per 28 L of culture broth. The pellet of the cyanobacteria was stored at −80° until required.

For purification of hydrogenase, the preserved cells (8.9 g wet wt) were thawed and then suspended in 50 mM Tris–HCl buffer, pH 7.5 containing 1 mM DTT. All buffer solns used during purification procedures contained 1 mM DTT. The suspension was sonicated at 70 W for 15 min and then centrifuged at 2400 *g* for 10 min; the supernatant was then centrifuged at 110000 *g* for 1 hr. After centrifugation, the supernatant was dialysed overnight against 1 mM Tris–HCl buffer, pH 7.5 and then applied to a 2.5 × 15 cm DEAE-cellulose column pre-equilibrated with 1 mM Tris–HCl buffer, pH 7.5 and eluted by 10 mM Tris–HCl, 50 mM Tris–HCl, pH 7.5 and then a step-wise gradient of NaCl in 50 mM Tris–HCl buffer. The active hydrogenase fractions were combined and concd using an Amicon PM-10 membrane filter and applied to 2.5 × 50 cm column of Sephacryl S-200 pre-equilibrated with 50 mM Tris–HCl buffer, pH 7.5 containing 0.2 M NaCl. Elution was carried out using the same buffer soln. Active fractions were pooled and concd by an Amicon PM-10 filter and stored at −80° until use as partially purified hydrogenase. The protein concn in CC eluants was continuously monitored at A_{280}. Sonication, centrifugation, dialysis and CC procedures were carried out in conditions as anaerobic as possible.

A crude fraction of ferredoxin was prepared according to ref. [22].

Hydrogenase activity was determined by measuring H_2 evolution from methyl viologen reduced by dithionite using a hydrogen electrode as reported previously [18]. The int. vol. of the reaction vessel was 2.24 ml. The reaction mixture contained 1 mM methyl viologen, 5 mM sodium dithionite and 50 mM buffer (50 mM Tris–HCl, pH 7.5 for activity survey during purification or 50 mM sodium phosphate, pH 7 for characterization of the enzyme). The assay temp. was 25°.

Protein determination was according to Peterson's modification of Lowry's method [23]

Acknowledgements We thank Dr M M Watanabe, National Institute for Environmental Studies, for providing the cultures of *M aeruginosa*, Dr T Fujii, Department of Agricultural Chemistry, Chiba University, for his critical reading of the manuscript and M Uebayasi of our Synthesized Material Fermentation Division for the loan of the hydrogen electrode system This work was supported by the Special Coordination Funds for Promoting Science and Technology, Japan.

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