CHARACTERIZATION AND PARTIAL PURIFICATION OF THE REVERSIBLE HYDROGENASE OF *ANABAENA CYLINDRICA*

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1. Introduction

Three types of hydrogenases are recognized in the literature; a reversible hydrogenase which has been highly purified and characterized from saccharolytic bacteria [1], an uptake hydrogenase which is seemingly incapable of evolving hydrogen and differs from the reversible hydrogenase in being saturated at low pH_2 [2], and the ATP-driven hydrogen evolution catalyzed by nitrogenase in the absence of other reducible substrates [3]. Species of cyanobacteria such as *Anabaena cylindrica* contain all three types of hydrogenase under some conditions [4].

Hydrogen evolution by nitrogenase in this organism has been studied in some detail [5–7]. The presence of an uptake hydrogenase, similar to the *Azotobacter* hydrogenase, has been demonstrated [8–10]. The presence of reversible hydrogenase activity can be directly shown with cultures of *A. cylindrica* that lack nitrogenase activity either through growth in the presence of tungsten instead of molybdenum, or through ammonium repression [11,12]. Hydrogen is produced by isolated heterocysts or vegetative cell fragments supplied with artificial electron donors [13]. In [14,15] crude extracts with hydrogenase activity were prepared from nitrate-grown cultures of *A. cylindrica* incubated anaerobically in the dark under an atmosphere of hydrogen. It was found that phenazine methosulphate, methylene blue, dichlorophenol-indophenol and toluidine blue were effective as electron acceptors, but the results suggested that redox coupling between the hydrogenase and ferredoxin had a very low efficiency. When crude extracts were prepared by homogenization, the hydrogenase was associated with a particulate fraction capable of reducing nitrate. Here we report on the preparation of a reversible hydrogenase from nitrogen-starved cultures of *A. cylindrica* and describe some of the properties of the partially purified enzyme. Our results indicate that this enzyme is quite different from hydrogenases isolated from other sources.

2. Materials and methods

2.1. *Cultures and growth conditions*

Anabaena cylindrica (629) cultures were grown as 48 l batch cultures in half-strength modified Allen and Arnon media [6]. After innoculation with a fairly dense innoculum, the cultures were grown at low light intensities for 24–48 h. After that time, the illumination was 4.6×10^4 ergs/cm^2/s. The cultures were sparged with a gas mixture of 0.3% carbon dioxide and 99.7% argon, 24–36 h before harvesting. Cultures were harvested with a Sharples centrifuge, and the paste was quick frozen and stored in liquid nitrogen until needed.

2.2. *Preparation of crude extract and purification steps*

Hydrogenase was obtained as a by-product during the purification of nitrogenase. The procedures will be detailed elsewhere. The paste was thawed in anaerobic, pH 8.25, buffer that was 40 mM TES (*N*-Tris-[hydroxymethyl] methyl 1-2 amino ethane sulfonic acid) 10 mM $MgCl_2$, 5 mM sodium dithionite and con-

tained 20 μg/ml each of DNase and RNase. The solution was sonicated anaerobically for 6 min. Protamine sulfate, 2%, 0.0034 ml, was added per mg protein in the extract (determined by the Lowry procedure [16]) and the resulting solution centrifuged at 30 000 × *g* for 20 min. The supernatant was loaded on a 2.6 × 10 cm DEAE column pre-equilibrated with pH 7.5 buffer (20 mM TES, 15 mM $MgCl_2$ and 2 mM dithionite). After washing the column with equilibrating buffer, hydrogenase was eluted with reverse pH 7.5 buffer (20 mM TES, 60 mM $MgCl_2$, 2 mM dithionite) flow and collected anaerobically. After desalting and concentration, the hydrogenase was further purified on a 2.6 × 55 cm G-200 column developed with pH 7.5 buffer (20 mM TES, 5 mM $MgCl_2$, 2 mM dithionite).

2.3. *Hydrogenase activity*

Fernbach flasks, 5 ml, fitted with serum stoppers were made anaerobic by alternately evacuating and pressurizing with argon. The flasks were vented to atmospheric pressure before the injection of any gases. Just prior to the addition of 0.20 ml buffered solution of sodium dithionite (25 mM in dithionite, 30 mM in Hepes, pH 7.8) the flasks were again vented to atmospheric pressure. A 20 mM Methyl viologen solution, 50 μl, was added as were varying amounts of water (bringing each assay to a final 1 ml). The assay was initiated with the addition of the appropriate volume of enzyme solution and ran for 20 min at 30°C on a thermostated shaking (150 rev./min) water bath. The reaction was terminated with the addition of 0.20 ml 25% trichloroacetic acid (TCA). Hydrogen produced was quantitated by injection of a 1.0 ml aliquot in a Varian gas chromatograph equipped with a thermal conductivity detector.

3. Results

3.1. *Crude extract activities*

Cell-free extracts evolved hydrogen from dithionite only when methyl viologen was added (table 1). Similar results have been obtained with the hydrogenase of some rumen bacteria [17]. Presumably the natural electron carrier to hydrogenase present in these extracts is not reduced by dithionite since hydrogen evolution can be driven by pyruvate (rumen bacteria). The natural electron carrier to the algal hydrogenase is unknown at present, and there is good evidence that its reduction by plant-type ferredoxins, including one that can be isolated from *A. cylindrica*, is sluggish at best. That hydrogenase is not reduced by spinach chloroplasts and *Anabaena* ferredoxin was reported [14] and confirmed in this laboratory. It seems that there is no carrier in the crude extract that can link the hydrogenase with the endogenous NADPH-dependent diaphorase (which normally reacts with ferredoxin). However, methyl viologen is an extremely effective electron carrier to the algal hydrogenase. The K_m for methyl viologen-mediated hydrogen evolution, 53 μM, is similar to that found for ferredoxin with *C. pasteurianum*, 100-fold less than the K_m for methyl viologen in that organisms [1] and 10-fold less than that found for *D. vulgaris* [18]. The K_m was derived from a reciprocal plot with 6 substrate concentrations (in the 200–10 μM methyl viologen range).

Acetylene has been reported to inhibit hydrogenase activity in both cultures and cell extracts of *K. pneumoniae* [19], *A. chroococcum* [19], and cultures of *A. cylindrica* [11]. However, with extracts of *A. cylindrica* and *C. pasteurianum*, we have found no inhibi-

Table 1
Crude extract activities under various conditions

	μl H_2/h/ml	% act.
Anabaena cylindrica		
Dithionite, methyl viologen	132	100
Dithionite	3.5	3
Pyruvate	2.2	2
NADPH	2.2	2
NADPH, methyl viologen	16.5	13
Dithionite, methyl viologen 7.2% C_2H_2	129	97
Dithionite, methyl viologen 12.5% C_2H_2	130	98
Dithionite, methyl viologen 24% C_2H_2	129	97
Clostridium pasteurianum		
Dithionite	567	100
Dithionite, methyl viologen	560	100
Dithionite, 12.5% C_2H_2	584	100

Reaction vials contained, as indicated, 5 mM dithionite (buffered solution), 1 mM methyl viologen, 0.5 mM NADPH and 2 mM pyruvate with 2×10^{-5} M thiamine pyrophosphate and 0.50×10^{-6} M CoA. Assays were begun with the addition of an aliquot of a crude extract prepared in the absence of dithionite

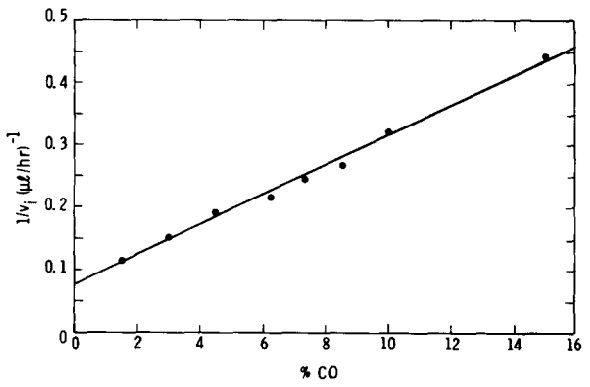

Fig.1. Stoichiometry of the reaction of hydrogenase with carbon monoxide. The enzyme solution used was a fraction from a G-200 column. The data were plotted according to Dixon. A linear plot indicates that one molecule of inhibitor is bound per enzyme independent site.

tion by acetylene of the hydrogenase enzyme of these organisms (table 1). As has been found for other hydrogenases, carbon monoxide was inhibitory. Substantial inhibition required 20-fold greater CO concentrations than found for the *C. pasteurianum* enzyme [20] with 0.045 atmospheres causing 50% inhibition. A plot of $1/v$ versus inhibitor concentration (fig.1) was linear, indicating that one molecule of carbon monoxide was bound per enzyme-independent site, similar to the *C. pasteurianum* enzyme.

Anabaena hydrogenase was not inhibited by α,α-bipyridyl, *o*-phenathroline or iodoacetate (all reagents at a final 10^{-3} M). That iron chelating agents have no effect on hydrogenase activity suggests that in the native enzyme the iron is firmly bound. Similar results have been obtained with *Chromatium* hydrogenase [21]. *Anabaena* hydrogenase was only slightly sensitive to oxygen inactivation, with the loss of only about 15% of initial activity after 90 min exposure to air.

3.2. *Purification*

The hydrogenase was readily solubilized by sonication. Addition of protamine sulphate resulted in the removal of some contaminating proteins and the photosynthetic lamellae (table 2). The hydrogenase could be further purified at this stage using either polyethylene glycol or further additions of protamine sulphate. When the supernatant was loaded on a short DEAE column (2.6 X 3 cm) equilibrated with 20 mM TES, 15 mM $MgCl_2$, the hydrogenase was absorbed, thus freeing it of most of the phycobiliproteins and a *c*-type cytochrome. The hydrogenase was eluted with 60 mM $MgCl_2$ using reversed buffer flow and desalted with G-25 Sephadex. Gel filtration of the hydrogenase fraction on G-200 effected further purification. The hydrogenase activity chromatographed with an app. mol. wt 230 000. (The column was calibrated with blue dextran, catalase, hemoglobin and cytochrome *c*, and K_d values calculated from elution volumes.) At present it is not known whether this represents the true molecular weight of this hydrogenase which would make it the largest reported hydrogenase, or if it indicates that the hydrogenase is bound in a complex with other proteins [22]. At this stage the hydrogenase had been purified 30-fold (see table 2) with a reasonable recovery of initial activity. Preliminary experiments indicate that further purification of the G-200 fraction is possible using gradient elution on a DEAE column. Elution of the hydrogenase required high ionic strengths (75 mM $MgCl_2$) indicating that it is a very acidic protein.

Table 2
Purification of hydrogenase

	Spec. act. (µl H_2/mg/h)	Purificn.	Recovery
Crude extract	13.2	1 X	100%
Protamine sulphate fractionation	37.9	3 X	100%
DEAE chromatography	149	11 X	86%
Gel filtration with G-200	395	30 X	30%

4. Discussion

Anabaena hydrogenase, although similar in some respects to previously characterized hydrogenases, has been found to have several unique, distinguishing characteristics. Among these are its relative insensitivity to carbon monoxide, inhibition stability in air, and facility of reaction with methyl viologen. Another unusual characteristic is the failure of this hydrogenase to interact with *Anabaena* ferredoxin. The nitrogenase of this organism has been shown [23–25] to be effectively reduced by ferredoxin in vitro and it is believed that the principle mechanism of nitrogenase reduction in vivo involves ferredoxin-catalyzed reactions. These results suggest that in *Anabaena cylindrica* there is a compartmentalization of reductant pools, and that this compartmentalization is effectively maintained by electron carrier specificity. Further study, both biochemical (more complete molecular characterization, and identification of the natural electron carrier) and physiological, will be necessary for an understanding of the role of this hydrogenase in the metabolism of cyanobacteria.

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