

Reversible hydrogenase of *Anabaena variabilis* ATCC 29413: catalytic properties and characterization of redox centres

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Abstract The catalytic and spectroscopic properties of the reversible hydrogenase from the cyanobacterium *Anabaena variabilis* have been examined. The hydrogenase required reductive activation in order to elicit hydrogen-oxidation activity. Carbon monoxide was a weak ($K_i = 35 \mu\text{M}$), reversible and competitive inhibitor. A flavin with the chromatographic properties of FMN, and nickel were detected in the purified enzyme. *A. variabilis* hydrogenase exhibited electron paramagnetic resonance (EPR) spectra in its hydrogen-reduced state, indicative of [2Fe-2S] and [4Fe-4S] clusters. Although no EPR signals due to nickel were detected, the results are consistent with the enzyme being a flavin-containing hydrogenase of the nickel-iron type.

Key words: Hydrogenase; EPR spectroscopy; Iron-sulfur protein; Nickel; Flavoprotein; Cyanobacterium

1. Introduction

Hydrogenases are hydrogen-activating enzymes which are widespread among microorganisms of various taxonomic groups. Depending on the metal content in the active centre hydrogenases may be divided into different classes [1,2]. The [Fe]-hydrogenases contain only iron-sulphur clusters, including a special catalytic 'H' cluster [1]. The [NiFe]-hydrogenases typically have two subunits of about 60 and 30 kDa; they contain nickel, in a dinuclear Ni-Fe site, and also [4Fe-4S], and sometimes [3Fe-4S], clusters [3]. Some hydrogenases have additional subunits containing prosthetic groups such as flavin or haem, which are required for interactions with electron acceptors or donors [4].

Heterocystous (nitrogen-fixing) cyanobacteria are capable of synthesizing two functionally different types of hydrogenases: uptake hydrogenases, which preferentially consume hydrogen, and reversible hydrogenases, which catalyze both H_2 uptake and evolution [5]. The uptake hydrogenases appear to be associated with nitrogen fixation [6]. The reversible hydrogenases of cyanobacteria are active under anaerobic conditions [7], and are implicated in hydrogen evolution [8,9]. In-

tact cells of three species of *Anabaena* were shown to evolve H_2 under anaerobic conditions in the dark, in the presence of carbohydrates [10]. This process was reversibly suppressed by light, and was attributed to the reversible hydrogenase. It was clearly distinct from hydrogen production by nitrogenase, which only occurs in the light, since in these experiments the cells were grown with ammonia and did not have nitrogenase activity. The rate of hydrogen formation by the cells was observed to correlate with their levels of hydrogenase activity as measured by H_2 evolution with reduced MV as electron donor [10].

Reversible hydrogenases have been isolated from a number of cyanobacteria [11]. They are characterized by their sensitivity to O_2 , thermotolerance and high affinity for H_2 . However their molecular properties are not well characterized. A hydrogenase preparation from *Spirulina maxima* was reported to be a monomer of 56 kDa [12], while one from *Anabaena cylindrica* consisted of two nonidentical subunits of 42 and 50 kDa [13]. The reversible hydrogenases from *Anacystis nidulans* and *A. variabilis* have been reported to have dimeric structures [14,15]. The sequence reported for the small subunit of *A. cylindrica* reversible hydrogenase showed no homology with those of any other hydrogenases [16].

More recently, the gene sequence of the hydrogenases of *An. nidulans*, *A. cylindrica* and *A. variabilis* have been determined [17]. Unexpectedly, they were found to be homologous with those of the more complex NAD(P)⁺-linked hydrogenases from *Alcaligenes eutrophus* and *Nocardia opaca* [18]. The latter belong to the [NiFe]-hydrogenases [18], but contain two extra 'diaphorase' subunits which bind FMN and iron-sulphur clusters, including, unusually, [2Fe-2S] clusters. Crude extracts of *An. nidulans* were shown to be able to evolve molecular hydrogen with NADPH as donor [17]. So far, no information on the prosthetic groups of cyanobacterial hydrogenases has been reported. In this work we have examined the catalytic properties and redox centres of the reversible hydrogenase isolated from the filamentous cyanobacterium *A. variabilis*.

2. Materials and methods

2.1. Organism and growth conditions

Axenic non- N_2 -fixing cells of *A. variabilis* ATCC 29413 [19] were grown photoautotrophically under continuous illumination in mineral medium [20] supplemented with 8 mM phosphate, 1.5 μM nickel chloride and 5 mM ammonium chloride. Growing cells were continuously sparged with air containing 1% CO_2 (300 ml/min). To induce reversible hydrogenase activity, the cell suspensions (7–10 μg chlorophyll/ml) were sparged with argon containing 1% CO_2 (300 ml/min) for 48 h.

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Abbreviations: *A.*, *Anabaena*; *Al.*, *Alcaligenes*; *An.*, *Anacystis*; BV, benzyl viologen; EPR, electron paramagnetic resonance spectroscopy; Mes, 2-(*N*-morpholino)ethanesulphonate; MV, methyl viologen.

2.2. Determination of hydrogenase activity

Hydrogen-evolution activity was determined by gas chromatography with MV^{+} as electron donor, at 30°C [21]. Hydrogen-uptake activity was determined spectrophotometrically by monitoring H_2 -dependent MV^{2+} reduction at 30°C in 20-ml anaerobic cuvettes containing a 2-ml liquid phase (50 mM Tris-HCl, pH 8.0, 1 mM MV , 0.05 mg hydrogenase) and 100% H_2 as gas phase. Assays were initiated by addition of the enzyme [22].

2.3. Hydrogenase purification

Reversible hydrogenase was purified by a method developed from that of Serebryakova et al. [15]. Cells from 40-l cultures of *A. variabilis* were harvested by centrifugation and resuspended in 200 ml of water. Cooled acetone (1.8 l) was added to the cell suspension under continuous stirring and the mixture was incubated for 30 min at 4°C. The pellet obtained by filtration through a porous glass filter was washed several times with cooled acetone and dried to a powder. The acetone powder was suspended in 400 ml of 50 mM phosphate, pH 7.0/2 mM dithiothreitol and the suspension was shaken overnight. The insoluble material was removed by centrifugation (10 000 × g, 40 min). The supernatant was applied to a 3.5 × 10 cm column of DEAE-Sephacel (Pharmacia) equilibrated with 50 mM phosphate buffer, pH 7.0, and washed with 50 mM Tris-HCl, pH 8.0, until the blue color was eluted. The hydrogenase was eluted with 50 mM Tris-HCl/500 mM NaCl in a total volume of 50 ml. The solution was desalted on a Sephadex G-25 column equilibrated with 20 mM Tris-HCl, pH 8.0, then applied to a 1.0 × 10 cm column of DEAE-cellulose (Whatman DE-52) equilibrated with the same buffer containing 0.1 M NaCl. The enzyme was eluted in a linear gradient of 0.1–0.5 M NaCl. The hydrogenase-containing fractions were pooled and applied to a Phenyl Sepharose CL-4B (Pharmacia) column, equilibrated with 20 mM Tris-HCl/0.35 M NaCl. The hydrogenase-containing fractions were pooled, concentrated on a small DE-52 column (0.5 × 4 cm) and applied to a 2.5 × 70 cm column of Sephacryl S-300 (Pharmacia). The fractions containing hydrogenase activity were pooled and concentrated using Centricon-10 microconcentrators (Amicon).

The purified enzyme had a specific activity in the range 7–10 $\mu\text{mol } H_2/\text{min} \cdot \text{mg protein}$ in the standard hydrogen-evolution assay. Electrophoresis in 7.5% polyacrylamide gel [23] showed a single band with R_f 0.65, when stained for hydrogenase activity, which coincided with the major band stained with Coomassie Blue. The degree of purity of the protein was estimated to be 95%.

2.4. Incubation conditions during activation

Each hydrogenase preparation ($\approx 1 \text{ mg/ml}$) was placed in the side-arm of a 20 ml glass vessel deoxygenated by five cycles of evacuation and re-equilibration with hydrogen or argon. 1 ml of 0.1 M $Na_2S_2O_4$ in 50 mM Tris-HCl, pH 8.0, was added to the main part of the vessel as an O_2 scavenger. At intervals during incubation under the appropriate gas phase, samples of hydrogenase were transferred into reaction cuvettes, and the activity was measured under standard conditions.

2.5. Analysis of flavin content

The protein was precipitated with trichloroacetic acid as previously described [24]. The presence of a flavin group in the supernatant was detected by optical absorption and by fluorescence. For the identification of the flavin, neutralized trichloroacetic acid supernatants were separated by HPLC with a Kontron system liquid chromatograph, using an Aquapore AX-300 C18 7 μm column (250 mm × 7 mm) in 0.1 M ammonium acetate : methanol. Detection was made with excitation at 450 nm and emission of fluorescence at 530 nm. Riboflavin, FMN and FAD standards (Sigma) were used to calibrate the column.

2.6. EPR characterization

EPR samples were prepared by diluting oxidized enzyme with buffer (50 mM Mes, pH 6.5), and concentrating by ultrafiltration through Centricon 30 microconcentrators (Amicon), at 4°C. Hydrogenase was activated in an EPR tube by reduction under oxygen-free, water-saturated hydrogen gas at room temperature for 16 h. The EPR tube was frozen and samples were stored in liquid nitrogen until use.

EPR spectra were recorded on a Bruker ESP300 spectrometer with an Oxford Instruments ESR900 helium flow cryostat. Spin quantitations of the EPR signals were determined by double integration of spectra recorded at 70, 30 and 10 K under non-saturating conditions.

3. Results and discussion

3.1. Kinetic properties of the purified hydrogenase

The reversible hydrogenase isolated from *A. variabilis* cells catalyzed H_2 evolution with MV^{+} as electron donor, at a maximum rate of 10.2 $\mu\text{mol } H_2/\text{min} \cdot \text{mg protein}$. The reaction occurred at constant rate and had no lag phase. As the enzyme was purified, its stability decreased, and its kinetic properties were altered. The half-time of hydrogenase inactivation when stored in the air at 4°C was 7 days in cell-free extracts, whereas that of the purified enzyme under similar conditions was 26 h. The temperature dependence of the enzyme-catalyzed reaction indicated an activation energy for the process of 65.8 kJ/mol at the optimal pH, 6.9 for the cell-free extract, while the value for the purified enzyme was 40 kJ/mol. The K_m for MV^{+} estimated for the cell-free extract was $170 \pm 10 \mu\text{M}$, while that of the purified enzyme was $55 \pm 5 \mu\text{M}$. These differences are possibly due to removal of other interacting proteins.

In the H_2 -oxidation assay with MV^{2+} or other redox dyes as acceptors, the purified enzyme initially showed no activity. Like other hydrogenases purified under aerobic conditions, it required reductive activation [22]. The kinetics of reductive activation and oxidative deactivation of hydrogenase were examined, using as an assay the H_2 -dependent reduction of MV^{2+} . The dithionite-activated process, under H_2 atmosphere, was rapid, with a stable activity level being reached in the first 15 min (Fig. 1a). Almost identical kinetics were observed with dithionite under an argon atmosphere. A similar effect of a strong reductant has been observed in the activation of the soluble hydrogenase from *Al. eutrophus* [25,26]. The *A. variabilis* enzyme was also activated in the presence of H_2 alone. This process was slower and exhibited a lag and a fast phase comparable with that reported for the hydrogenase of *Desulfovibrio gigas* [27]. After activation, *A. variabilis* hydrogenase remained active under Ar atmosphere, but rapidly lost its activity when exposed to air (Fig. 1b). The hydrogenase oxidized by air could be reactivated by H_2 without a significant loss of activity (data not shown). These results may be compared with the flavin-containing hydrogenase of *Al. eutrophus*, which, when activated, was found to lose activity rapidly in the absence of H_2 [26].

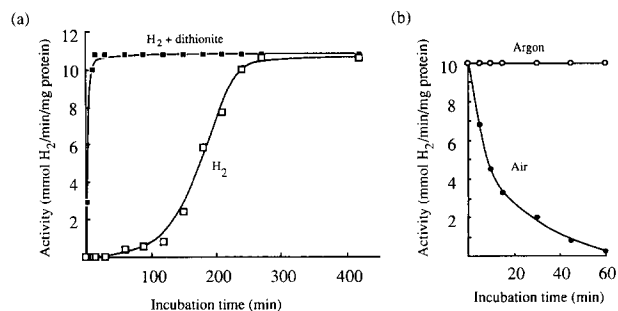


Fig. 1. (a) Reductive activation of *A. variabilis* hydrogenase. Samples of anaerobic solution of enzyme (1 mg/ml protein) were incubated with 1 mM sodium dithionite under H_2 (■) or under H_2 without other reducing agent (□). At the indicated times 50 μl of sample were removed and their activities determined as MV -dependent H_2 oxidation. (b) Oxidative inactivation of activated H_2 -oxidizing activity of *A. variabilis* hydrogenase. Samples of hydrogen activated enzyme were incubated under air (●) or Ar (○). Activities of samples were determined as MV -dependent H_2 oxidation.

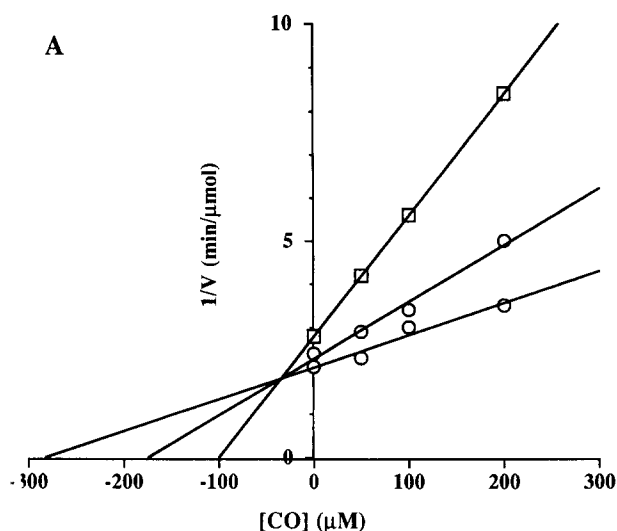


Fig. 2. Dixon plot of carbon monoxide inhibition of H_2 -oxidizing activity of *A. variabilis* hydrogenase. The concentrations of H_2 and CO were adjusted by adding calculated volumes of CO to H_2 -Ar mixture through a gas-tight syringe. (■) 5% H_2 , (○) 10% H_2 , (●) 20% H_2 .

The activated hydrogenase catalyzed H_2 oxidation with a number of artificial electron acceptors under H_2 atmosphere, the most effective being viologen dyes. The reaction rates were approximately equal for MV^{2+} and BV^{2+} (9.0–10.4 $\mu\text{mol}/\text{min}\cdot\text{mg}$ protein). The K_m values measured for the two oxidized viologens in the H_2 -uptake reaction were similar (K_m MV^{2+} = 250 ± 6 μM ; K_m BV^{2+} = 226 ± 10 μM), but higher than those for the reduced form of MV^+ in the H_2 evolution assay. The maximal rate of H_2 oxidation was observed at pH 8.0–8.5. The K_m for H_2 of the enzyme estimated in the reaction of MV^{2+} reduction was 11.3 μM . This value is comparable to that reported for the reversible hydrogenases of other cyanobacteria [5].

The activated hydrogenase was able to reduce plant-type ferredoxin isolated from the same species, and NAD^+ , under hydrogen atmosphere. However, the rates were very slow, and no hydrogen formation was detected with either reduced NADP or ferredoxin, in contrast to cell extracts from *An. nilulans*, which performed NADP-dependent hydrogen evolution [17].

3.1. Effect of carbon monoxide

Carbon monoxide was inhibitory to both H_2 oxidation and evolution. The inhibition was completely reversed by removing CO, but not affected by light. The dependence of the H_2 oxidation rate on CO concentration indicated that the inhibition was competitive with respect to H_2 . The K_i for CO was estimated to be 35.5 μM (Fig. 2), indicating a relatively low affinity for this inhibitor. This value is within the range reported for other [NiFe]-hydrogenases, whereas the K_i values of the [Fe]-hydrogenases for CO are of the order of 1 μM [1].

3.2. Flavin and metal content

The presence of flavins linked noncovalently to the protein was investigated. Noncovalently bound groups were extracted by precipitating the apoprotein fraction with trichloroacetic acid. The presence of a flavin cofactor in *A. variabilis* hydrogenase was detected by its characteristic fluorescence spectrum

with an emission maximum at 530 nm when excited at 450 nm. The flavin group was identified by its retention time on HPLC. In duplicate determinations the elution volume of the enzyme cofactor was close to that of FMN. The results were inconsistent with the flavin being FAD, but could not exclude other, unknown flavins.

Preliminary analysis of a sample of the enzyme by atomic absorption spectrophotometry (M. Medina, unpublished) demonstrated the presence of nickel and iron in the ratio 1 g atom Ni:34 g atom Fe.

3.4. Electron paramagnetic resonance spectroscopy

As isolated, under air atmosphere, *A. variabilis* hydrogenase exhibited a nearly isotropic EPR signal, with features at $g = 2.019$, 2.006 and 2.002 (not shown). Upon reduction under hydrogen atmosphere, or with sodium dithionite under Ar, this isotropic $g = 2.01$ signal decreased in intensity until it disappeared. The shape of this signal was similar to the one found for the [3Fe-4S] cluster in *D. gigas* ferredoxin II [28]. Spin quantitation of this signal yielded approx. 10% of the signals observed in the reduced state (see below), indicating that it is a minority species, in comparison with the strong

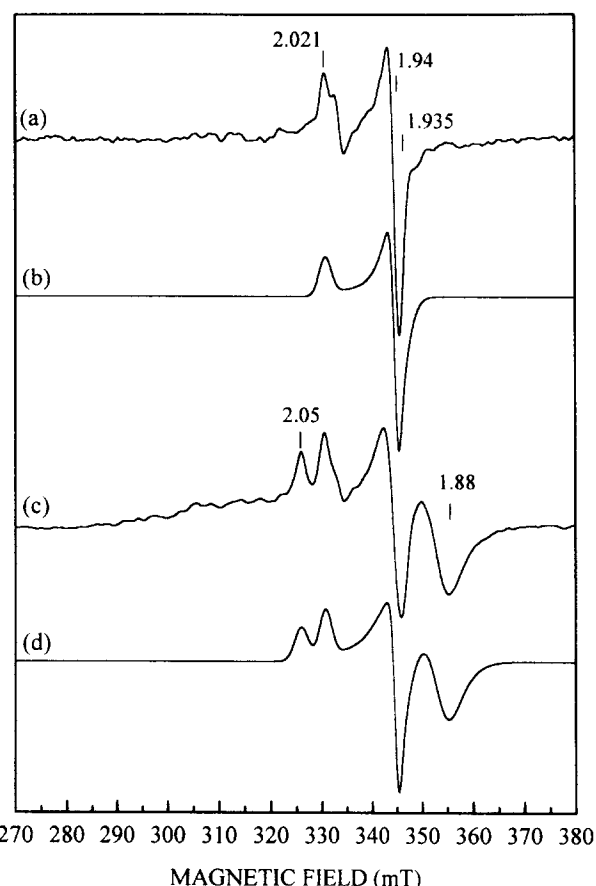


Fig. 3. EPR spectra of the reduced states of *A. variabilis* hydrogenase, in 20 mM Mes pH 6.5. (a) Spectrum recorded at 30 K after reducing with hydrogen for 16 h at room temperature and flushing the sample with argon for 3 min to remove hydrogen; (b) simulation of spectrum (a); (c) same as (a) but recorded at 10 K; (d) simulation of spectrum (c). Other EPR conditions: microwave power (a) 2 mW, (c) 20 mW. The magnetic field was modulated at 100 kHz and with 1.0 mT amplitude. The microwave frequency was 9.36 MHz. The receiver gain for spectrum (a) is twice that for spectrum (c).

signal from the [3Fe-4S] cluster of *D. gigas* hydrogenase [28], which is definitely a component of the enzyme [3]. Similar $g = 2.01$ signals in substoichiometric amounts have been reported for the [NiFe]-hydrogenase from *Al. eutrophus* [29]. In view of the low amount of the signal and the fact that [4Fe-4S] clusters can be converted into [3Fe-4S] clusters under oxidising conditions [30], it seems likely that the presence of this signal in the isolated *A. variabilis* enzyme represents oxidatively damaged [4Fe-4S] clusters. In support of this, we observed that the intensity of the signal doubled after reoxidation with air of a sample previously reduced under hydrogen atmosphere.

Prolonged incubation of *A. variabilis* hydrogenase under hydrogen induced the appearance of a sharp EPR signal with resonances at $g = 2.021$, 1.94 and 1.935 (Fig. 3). This signal could be observed at temperatures below 70 K, broadening beyond detection above 80 K. The g factors, line shape, and temperature dependence of the observed EPR signal are consistent with the presence of a single type of reduced [2Fe-2S] cluster. This was the only signal evident at temperatures down to 20 K, but upon lowering the temperature of the reduced sample to 10 K, two additional EPR resonances were observed at $g = 2.05$ and 1.88, from a second paramagnetic centre. These signals were insensitive to power saturation at 10 K. This is consistent with reduced [4Fe-4S] clusters, which have a more rapid spin relaxation rate than [2Fe-2S] clusters [31,32]. The spectrum became broader at 5 K, but no additional low-field resonances were detected for the reduced or the oxidized enzyme at temperatures between 5 and 100 K. The same signals were detected upon reduction of the enzyme with sodium dithionite. The EPR signals from the reduced hydrogenase disappeared upon progressive incubation under argon atmosphere, giving an EPR-silent state. There was no evidence of the rhombic EPR signals from nickel seen in some [NiFe]-hydrogenases in either the oxidized, the fully reduced or the argon-exchanged reduced states; nor of the H-cluster signals observed in [Fe]-hydrogenases. It may be noted that several [NiFe]-hydrogenases have been reported which do not exhibit EPR signals from nickel, including *N. opaca* [33] and *Pyrococcus furiosus* [34].

Treatment with carbon monoxide did not produce any detectable changes in the EPR spectra of either the oxidized or reduced states of *A. variabilis* hydrogenase. No trace of the characteristic signals of the CO-derivatives of [Fe]-hydrogenases [1] was observed. Reoxidation under air atmosphere and re-reduction under hydrogen produced the same signals as before for the reduced enzyme.

The present work on *A. variabilis* reversible hydrogenase has demonstrated the presence of at least a [2Fe-2S] and a [4Fe-4S] cluster. Preliminary analyses are consistent with the presence of nickel and FMN. These results are compatible with the gene sequence of hydrogenase from the cyanobacterium *An. nidulans* [17], which indicates homology with the hydrogenase of *Al. eutrophus*. The latter enzyme contains nickel and FMN, and has a high content of iron and sulphide, including at least one [2Fe-2S] cluster [18,29].

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