Role of hydrogen in the activation and regulation of hydrogen oxidation by the soluble hydrogenase from *Alcaligenes eutrophus* H16

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The activation kinetics of the H₂-oxidizing activity of the soluble hydrogenase from *Alcaligenes eutrophus* H16 were investigated. Activation with Na₂S₂O₄ plus 101 kPa H₂ resulted in a rapid increase in activity over 1 h and constant activity after 3 h incubation. Less-stable activations were achieved if enzyme was incubated with Na₂S₂O₄ under 1 kPa H₂ or 101 kPa N₂. The enzyme could also be partly activated either with NADH alone or with H₂ alone. The level of activity obtained with both 101 kPa H₂ and NADH present was greater than that obtained with either 101 kPa H₂ or NADH alone. Activation with H₂ plus NADH was virtually independent of NADH concentration but highly dependent on H₂ concentration. The effects of various concentrations of H₂ and constant concentration of NADH on the level of activation were the same whether H₂ oxidation was assayed by H₂-dependent Methylene Blue or NAD⁺ reduction. Diaphorase activity did not require activation and was little affected by the treatments that activated H₂-oxidizing activity. The results suggest that H₂ plays an important role in regulating the level of H₂-oxidizing activity in this soluble hydrogenase.

INTRODUCTION

* The soluble hydrogenase (H₂:NAD⁺ oxidoreductase, EC 1.12.1.2) from the hydrogen bacterium *Alcaligenes eutrophus* H16 is a complex tetrameric enzyme (Schneider & Piechulla, 1986). Each tetramer contains Fe–S centres, Ni and FAD as prosthetic groups (Hornhardt et al., 1986). The enzyme exhibits both NAD⁺-dependent H₂ oxidation and NADH dehydrogenase (diaphorase) activity under appropriate conditions. H₂ oxidation can also be assayed independently of NAD⁺/NADH binding by the use of artificial electron acceptors such as Methylene Blue (Schneider & Schlegel 1976).

The soluble hydrogenase from *A. eutrophus* H16, like many other aerobically purified hydrogenases, requires activation before H₂-oxidizing activity is observed. This soluble hydrogenase is remarkably stable towards O₂ when in the inactive, oxidized, state, but is rendered O₂-sensitive upon activation. Therefore the removal of O₂ as part of, or preceding, the activation is required (Schneider & Schlegel, 1976, 1981). The enzyme can be activated for H₂ oxidation (usually under anaerobic conditions) with H₂ alone (Egerer et al., 1982), NADH plus H₂, NAD⁺ plus H₂, Na₂S₂O₄, u.v. irradiation or glucose plus glucose oxidase (Schneider & Schlegel, 1976). The levels of activation achieved with these treatments have not been compared. A pivotal catalytic role for NADH in the activation of H₂-oxidizing activity has been suggested by Schneider and Schlegel (1976). When inactive enzyme is assayed with NAD⁺ plus H₂, a lag phase is observed before NAD⁺-dependent H₂ oxidation begins. This lag phase can be eliminated by including catalytic concentrations of NADH.

Once activated, this enzyme is reported ‘unusually’ unstable (Schneider & Schlegel, 1976). This instability accounts for up to a 19% loss of H₂-oxidizing activity after incubation for 24 h at 4°C in the presence of 101 kPa H₂ plus 25 μM-NADH (Schlegel et al., 1981). In the present paper we have compared the effects of some of the activators described above in terms of their effects on the stability of the H₂-oxidizing activity of the soluble hydrogenase from *A. eutrophus* H16. The results suggest that H₂ performs an important regulatory role in both the activation and stability of H₂-oxidizing activity.

MATERIALS AND METHODS

Purification of the soluble hydrogenase

The soluble hydrogenase was purified by using a modification of the method described by Schneider et al. (1983) in which the ion-exchange chromatography column was preceded by the Procion Red affinity column. Peak activity fractions eluted from the second column exhibited specific activities of 30–65 μmol of H₂ oxidized/min per mg of protein after activation for 2 h with 101 kPa H₂ plus 1 mM-Na₂S₂O₄ [spectrophotometric assay monitoring Methylene Blue reduction (85 μm) at pH 8.0 and 30°C]. Peak fractions were then dialysed at 4°C against 50 mM-potassium phosphate buffer, pH 7.0, and then stored under air at −20°C. After prolonged storage (>1 year), the enzyme was activated and reassayed as above and exhibited specific activities in the range 20–40 μmol of H₂ oxidized/min per mg of protein. SDS/polyacrylamide-gel-electrophoretic analysis of these samples revealed that the originally nearly homogeneous protein had undergone a limited degree of degradation during storage. Although the specific activities of these

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preparations are low relative to those described by Schneider et al. (1983), the experiments described in the present paper have made use of enzyme preparations of consistent activity so as to enable comparison between the various activation methods employed.

**Incubation conditions during activation**

Before activation the enzyme solution (1 mg of total protein/ml) was deoxygenated at room temperature by five cycles of evacuation and re-equilibration with O₂-free N₂. Unless otherwise stated, hydrogenase was then activated at room temperature in the inner chamber of a stoppered anaerobic double-chambered 13 ml serum-type incubation vial. To the outer chamber was added 0.5 ml of 0.1 M- Na₂S₂O₄ in 50 mM-Tris/HCl buffer, pH 8.0, to act as an O₂ scavenger. Additions to the enzyme solution were made from freshly prepared anaerobic stock solutions. Added volumes did not exceed 1% of the final volume. Mixtures of H₂ and N₂ in the incubation vials were generated by using a Matheson Dynablender gas mixer (Matheson Gas Products, Buffalo, NY, U.S.A.).

**Determination of hydrogenase and diaphorase activities**

Hydrogenase activity was determined by spectrophotometric assays monitoring either H₂-dependent Methylened Blue or NAD⁺ reduction; 50 mM-Tris/HCl buffer, pH 8.0, was used as buffer in all activity assays. H₂-dependent Methylened Blue-reducing activity was determined at 30°C in stoppered 6 ml cuvettes containing 3 ml of H₂-saturated buffer and Methylened Blue (85 μM). Assays were initiated by addition of enzyme. Methylened Blue reduction was monitored spectrophotometrically by using an experimentally derived molar absorption coefficient (ε) of 32.8 mm⁻¹ cm⁻¹ at 600 nm. The H₂-dependent NAD⁺ reducing activity was assayed as described by Schneider & Schlegel (1976). The diaphorase activity of the enzyme was monitored as NADH-linked ferrocyanide reduction as described by Schneider & Schlegel (1976). In all assays the rate was determined from the initial rate of reaction (0–10 s). All absorbance changes were recorded on a Beckman DU-7 spectrophotometer.

**Chemicals**

All gases were of high purity (> 99.99%) and were obtained locally. Residual O₂ was stripped from these gases by passage through a heated Cu-based catalyst (R3-11: Chemical Dynamics Corp., South Plainsfield, NJ, U.S.A.). All other chemicals used were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.).

**RESULTS**

**Activation of H₂-oxidizing activity with Na₂S₂O₄**

The time courses for the activation of H₂-dependent Methylened Blue-reducing activity of the soluble hydrogenase when incubated with Na₂S₂O₄ under various gas phrases are shown in Fig. 1. Activity of initially inactive enzyme incubated with Na₂S₂O₄ (1 mM) under 101 kPa H₂ increased over the first 3 h to a stable level. The initial increase in activity of enzyme incubated with Na₂S₂O₄ under 1 kPa H₂ (balance N₂) was similar during the first 60 min, but then activity was slowly and continuously lost over the next 4.5 h. After 6 h the activity was 75% of the activity of enzyme activated under 101 kPa H₂.

Removal of the H₂ during this gradual loss of activity resulted in a more rapid loss of activity (Fig. 1). Enzyme activated with Na₂S₂O₄ in the presence of 101 kPa N₂ also lost activity at a similar rate after an initial activation phase. Addition of 1 kPa H₂ after 4 h stabilized the remaining activity but resulted in no regain of activity over the next 2 h. If 101 kPa H₂ was added, no reactivation of the enzyme could be detected within 1 h. However, up to 90% of the lost activity could be recovered after 12 h incubation. Additional experiments showed that the rate of activation and the final level of activity reached were independent of Na₂S₂O₄ concentrations from 0.01 to 2.5 mM (results not shown).

**Activation of H₂-oxidizing activity with NADH and H₂**

The time courses for the activation of H₂-dependent Methylened Blue-reducing activity of hydrogenase incubated with various concentrations of NADH with or without H₂ are shown in Fig. 2. For enzyme activation with NADH plus 101 kPa H₂, the rate of activation and final level of H₂-oxidizing activity were independent of NADH concentration, even when the NADH concentrations were approximately stoichiometric with enzyme. In the absence of H₂ (N₂ gas phase), activation with NADH resulted in an initial burst of activity, which declined to a lower, stable, activity after approx. 30 min. Activity was only 30% higher when enzyme was incubated with 50 μM-NADH rather than 0.5 μM-NADH. When enzyme was activated with H₂ (101 kPa) alone there was a lag phase of 30 min before the activity sharply increased from zero to a stable level. The final
activation was approximately twice that of enzyme activated with NADH alone, but only approx. 30% of that exhibited by enzyme activated with NADH plus 101 kPa H₂.

The hydrogenase was activated at the same rate and to the same final activity with either NAD⁺ or NADH when in the presence of H₂. However, in the strict absence of H₂ the hydrogenase was not activated by 50 μM-NAD⁺ alone (results not shown). The rate of activation with H₂ alone (no added NADH) was dependent on the protein concentration of the enzyme solution; increased protein concentrations resulted in a faster activation. The H₂ concentration also affected the activation; lower concentrations gave lower rates of activation than did higher concentrations. However, neither the protein concentration nor the H₂ concentration affected the final specific activity of the enzyme in the absence of NAD⁺/NADH (results not shown).

Interconversion of activated states

To determine if the activity state of hydrogenase produced under one set of conditions could be converted into another state by manipulation of incubation conditions, the experiment described in Fig. 3 was performed. Enzyme was activated with NADH plus various concentrations of H₂ and N₂ for 3 h. After this, the incubation conditions were altered and the effect on hydrogenase activity was determined. Enzyme activated with NADH plus 1 kPa H₂ reached a stable activity at 6 h that was about 70% of that obtained for the enzyme activated with NADH plus 101 kPa H₂. The removal of the H₂ from a duplicate incubation (1 kPa H₂) resulted in a non-linear loss of activity over the following 3 h. The activity did not tend towards zero but appeared to approach a stable lower level. Addition of Na₂S₂O₄ (1 mM final concentration) to hydrogenase initially activated with H₂ alone led to a rapid increase in activity that stabilized at the same level as that exhibited by hydrogenase originally activated with NADH plus 101 kPa H₂. For hydrogenase originally activated with NADH alone (N₂ gas phase), the low level of stable activity observed after 3 h was increased when the N₂ gas phase was replaced with H₂. The activity increased more slowly than with Na₂S₂O₄ but the final activity was again equivalent to that for enzyme originally activated with NADH plus 101 kPa H₂.

Effect of H₂ concentration on activation with NADH

Whereas activation with NADH plus H₂ appeared to be independent of NADH concentration, Fig. 3 suggested that the H₂ concentration could affect the final level of activity exhibited by this enzyme. To quantify this effect, hydrogenase activity was measured after incubation for 20 h in the presence of NADH plus H₂ pressures ranging from 0 to 101 kPa. Fig. 4(a) shows, like Figs. 2 and 3, that the hydrogenase could be activated in the presence of NADH alone (N₂ gas phase). As the H₂ concentration in the gas phase above the incubation was increased, the H₂-oxidizing activity also increased. Maximal activity was observed for enzyme activated in the presence of NADH plus 101 kPa H₂. NADH disappearance during such an incubation is less than 50% (final NADH concentration 28 μM). The maximal level of activity shown in Fig. 4(a) (NADH plus 101 kPa H₂) is somewhat less than that achieved during shorter incubations. This
suggests that prolonged incubation (> 6 h) under activating conditions can result in a partial loss of activity similar to that reported by Schlegel et al. (1981).

Fig. 4(b) shows the rates of H₂ oxidation exhibited when samples of hydrogenase activated with NADH plus 101 kPa H₂ for 20 h were evacuated (to remove the H₂), flushed with N₂ and then incubated for a further 20 h in vials containing 0–101 kPa H₂. The activity of these hydrogenase samples changed in response to the H₂ concentration in the second incubation. The activities exhibited for a given H₂ concentration in both Figs. 4(a) and 4(b) were similar, even at low H₂ concentrations.

The effects of activation of the H₂-oxidizing activity are apparently restricted to the portions of the hydrogenase that are involved in H₂ oxidation (Table 1). The differences in the rates of H₂-dependent Methylene Blue reduction exhibited by hydrogenase activated under various conditions used in Fig. 4 were also observed when H₂-oxidizing activity was assayed by monitoring NAD⁺ reduction. No H₂-oxidizing activity was observed for hydrogenase incubated under strictly anaerobic conditions for 20 h (no NADH, N₂ gas phase). However, after a short lag phase (up to 1 min) this apparently inactive enzyme started to exhibit H₂-dependent NAD⁺ reduction. This effect is presumably due to autocatalytic activation of the hydrogenase resulting from the generation of NADH in the assay cuvette. No such effect was observed in H₂-dependent Methylene Blue reduction assays. The various activation states were also observed with enzyme of substantially higher specific activity (Table 1, values in parentheses).

In contrast with the effects of H₂ and NADH on the H₂-oxidizing activity of the hydrogenase, incubation of enzyme with NADH in the presence or in the absence of H₂ had no significant effect on the diaphorase activity of the enzyme (Table 1). The diaphorase activity, unlike the H₂-oxidizing activity, did not require activation and exhibited maximal activity before any treatment used to activate H₂-oxidizing activity.

**Table 1. Effect of activation on H₂-oxidizing and diaphorase activity**

<table>
<thead>
<tr>
<th>Activation treatment</th>
<th>H₂-linked Methylene Blue reduction</th>
<th>H₂-linked NAD⁺ reduction</th>
<th>Diaphorase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>101 kPa N₂</td>
<td>0 (0.2)</td>
<td>0.2</td>
<td>53.2</td>
</tr>
<tr>
<td>101 kPa N₂ + 50 µM-NADH</td>
<td>3.1 (5.4)</td>
<td>4.0</td>
<td>50.5</td>
</tr>
<tr>
<td>1 kPa H₂ + 50 µM-NADH</td>
<td>6.1 (16.8)</td>
<td>5.8</td>
<td>46.7</td>
</tr>
<tr>
<td>101 kPa H₂ + 50 µM-NADH</td>
<td>12.7 (37.4)</td>
<td>12.3</td>
<td>46.6</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In this study we have characterized the role of H₂ in the regulation of both the activity and the stability of H₂ oxidation catalysed by the soluble hydrogenase from *A. eutrophus* H16. When H₂-oxidizing activity was activated by Na₂S₂O₄ plus H₂ (Fig. 1), the initial level of activation (up to 90 min) was independent of the H₂ concentration (either 1 or 101 kPa). The removal of H₂ from the 1 kPa H₂ incubation led to a progressive loss of activity, suggesting that H₂ is important in maintaining the activity in Na₂S₂O₄-activated enzyme. This point was emphasized by the activation conducted in the presence of 101 kPa N₂. The lower maximal activity of this incubation, the stabilization against the further loss of the residual activity by the addition of low concentrations of H₂ (1 kPa) and a long-term re-activation in the presence of high H₂ concentrations (101 kPa) all point to a role for H₂ in both the activation process and the maintenance of activity when hydrogenase is activated with the strong reductant Na₂S₂O₄.

Activation of hydrogenase with combinations of the physiologically relevant reductants, NADH and H₂, is
more complex. The enzyme can apparently be activated to three kinetically distinct states, namely the two dissimilar low-activity states achieved by activation with NADH or H₂ alone (Fig. 2) and a high-activity state achieved by activation of the enzyme by the more than additive effects of both NADH and H₂ (Figs. 2 and 3).

The pivotal catalytic role of NADH in the activation of this enzyme was described in the Introduction. Our results (Fig. 2) confirm this role and suggest that activation with NADH plus H₂ (101 kPa) is independent of NADH concentration between 0.5 and 50 μM. Furthermore, NAD⁺ could activate the enzyme in the presence of H₂ but not NADH. Our observation that the enzyme can be partially activated by NADH alone (Fig. 2) has not been reported before, although a modification of the e.p.r. signal by NADH alone was reported (Schneider et al., 1979). The fact that the soluble hydrogenase slowly evolves H₂ in the presence of NADH (0.16 μmol/min per mg of protein in the presence of 2.4 mM-NADH; Schneider & Schlegel, 1976) is unlikely to account for this activation, given the low NADH concentrations used. Furthermore, the small increase in activation (30%) by a 100-fold increase in NADH concentration (0.5–50 μM) and the distinctive NADH-linked activation kinetics compared with those observed with H₂ alone suggest that H₂ evolution does not contribute to the activation of enzyme incubated with NADH alone. These results imply that the activations by NADH and by H₂ are through separate mechanisms. Activation of this hydrogenase with H₂ alone has been reported by Egerer et al. (1982) with enzyme of specific activity comparable with that used in the present study (20 μmol of H₂/min per mg of protein). These workers observed a lag phase during activation similar to that observed in the present study (Figs. 2 and 3). Activation with H₂ alone did not occur with immobilized hydrogenase. An intermolecular electron-transfer reaction between H₂-activated and unactivated hydrogenase molecules was proposed, which is in agreement with our observation that the enzyme concentration affects the rate of activation, but not the final level of activity achieved after incubation with H₂ alone.

Activation of this hydrogenase with both H₂ and NADH is kinetically distinct from the activations discussed above and produces a level of activity greater than the sum achieved with either activator alone (Figs. 2 and 3). This observation suggests a third, distinct, activation mechanism involving the concerted effects of both H₂ and NADH. Experiments to interconvert the enzyme activity between the proposed distinct activity states (Fig. 3) provided three important results. First, the levels of activity achieved with either Na₂S₂O₄ plus 101 kPa H₂ or NADH plus 101 kPa H₂ were the same. Secondly, the level of activation in the presence of NADH and H₂ was dependent on the H₂ concentration. Thirdly, removal of H₂ from enzyme activated with NADH plus H₂ resulted in a loss of activity, which did not, however, tend to zero.

When referred to the first point, Schneider et al. (1979) observed that the e.p.r. signal of the enzyme (as isolated) can be modified by NADH alone. Addition of H₂ to the NADH-reduced sample leads to further modification of the signal that is almost identical with that observed for enzyme reduced directly with Na₂S₂O₄. Apparently, both forms of reduction lead to an equivalent fully reduced form of the enzyme. Our results indicate that activity measurements can be correlated with these biophysical observations; e.g. NADH-plus-H₂-treated hydrogenase oxidizes H₂ at the same rate as Na₂S₂O₄-plus-H₂-treated enzyme.

The effects of H₂ concentration on activation and stabilization were further characterized (Fig. 4). The removal of H₂ (but not NADH) from an NADH-plus-H₂-activated enzyme resulted in a decrease in enzyme activity to a stable level approximately equivalent to, but no less than, that achieved for hydrogenase activated with NADH alone. Apparently, the low level of activation achieved with NADH alone 'primed' the enzyme so that it can respond to the ambient concentration of H₂. The inter-relationship between NADH and the ambient H₂ concentration may be of physiological significance in regulating the activity of the soluble hydrogenase from *A. eutrophus* H16 in *vivo*. Under natural conditions the soluble hydrogenase is likely to be exposed to continuously varying concentrations of both NADH and H₂. The activation level of the enzyme is rather insensitive to NADH concentration (Fig. 2) but is acutely sensitive to H₂ concentration (in the presence of NADH), especially in the region of the reported *Kₐ* for H₂, 37 μM (Schneider & Schlegel, 1976) (Fig. 4). Furthermore, the response time of the hydrogenase to changes in H₂ concentration is sufficiently slow so as to enable the activity level to change in response to progressive long-term changes in the ambient H₂ concentration and not merely short-term fluctuations.

Although the kinetic analyses we have described in this paper have not made use of hydrogenase of the highest specific activity described in the literature (Schneider et al., 1983), we have found that the activation effects obtained in Table 1 hold true for enzyme preparations of specific activity between 10 and 40 μmol/min per mg of protein (H₂-dependent Methylene Blue reduction) and for protein concentrations between 0.2 and 3.5 mg of total protein/ml (specific activity 20 μmol/min per mg of protein). Although it is possible that some of the effects that we have observed are due to the presence of contaminating proteins, it seems more plausible that our preparation contains inactive and/or degraded enzyme. Such protein is known to accumulate in whole cells and may not be completely removed by using affinity chromatography (Schneider et al., 1983). The presence of catalytically incompetent enzyme would be compatible with both the low specific activity of our preparation and the observation that, where overlap exists, our results agree well with previously published kinetic analyses of this hydrogenase. One point of disagreement is our observation that this hydrogenase cannot be activated under simply anaerobic conditions (Table 1). Schneider & Schlegel (1976) reported activation with a glucose/glucose oxidase system to achieve anaerobiosis. Perhaps the flavin-containing glucose oxidase acts as more than a simple O₂ scavenger and actively participates in the reduction of the hydrogenase, possibly through direct protein–protein interactions or loosely associated reduced flavin. It is of interest that Lissolo et al. (1984) were unable to activate the periplasmic hydrogenase from *Desulfovibrio gigas* after 12 h of flushing with O₂ and H₂-free N₂, whereas Berlier et al. (1982) were able to activate the same enzyme rapidly with a glucose/glucose oxidase system.

The apparent regulatory role of H₂ in the activation and maintenance of activity of *A. eutrophus* soluble
Hydrogenase is also of significance to inhibitor studies of this enzyme. On the one hand, inclusion of H₂ during the incubation with inhibitor may mask the effects of inhibitors competitive with H₂. On the other hand, removal of H₂ leads to decreases in activity not related to the inhibitor. During this study we observed that both C₂H₂ and NO could act as inhibitors of the soluble hydrogenase. Details of the effects of NO are described in the following paper (Hyman & Arp, 1988). With C₂H₂, transfer of NADH-plus-H₂-activated enzyme to an H₂-free C₂H₂-filled vial led to complete loss of activity within 4 h, whereas enzyme transferred to an H₂-free N₂-filled vial lost only 60% of its activity over the same period. No inhibition by C₂H₂ was observed in the presence of H₂. The demonstration that activity of the soluble hydrogenase is highly dependent on the ambient H₂ concentration, and the methods used to activate the enzyme, requires that careful consideration must be paid to the interpretation of results obtained with this enzyme in the course of inhibitor and stability studies.

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