The effect of re-oxidation on the reduced hydrogenase of *Desulfovibrio vulgaris* strain Hildenborough and its oxygen stability

Cees van Dijk, Anita van Berkel-Arts and Cees Veeger

Department of Biochemistry, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands

Received 29 April 1983

Upon reduction of the oxygen-stable hydrogenase of *Desulfovibrio vulgaris* strain Hildenborough this enzyme becomes oxygen sensitive. Only anaerobic oxidation of the reduced enzyme with 2,6-dichlorophenol-indophenol, in the presence of iron and EDTA, followed by addition of Tris–EDTA (pH 9.0) converts the enzyme back into its oxygen-stable state, without loss of activity.

Hydrogenase Desulfovibrio vulgaris *Re-oxidation* *Oxygen stability*

1. INTRODUCTION

One of the intriguing questions concerning the high activity, three (4 Fe–4 S) cluster-containing hydrogenases from different species is their stability in the presence of O_2. The enzyme of *Desulfovibrio vulgaris* strain Hildenborough [1] is oxygen-stable in its oxidized form, the hydrogenases from *Megasphaera elsdenii* [2] and *Clostridium pasteurianum* [3] are oxygen-sensitive, both in their oxidized and reduced forms. However, upon reduction the *D. vulgaris* enzyme becomes oxygen sensitive. When the reduced enzyme is re-oxidized, it does not convert back into its oxygen-stable form [4]. This suggests that the way by which this hydrogenase is isolated, by means of washing with Tris–EDTA (pH 9) probably in combination with factors present in the bacterium and/or growth medium, affect the transformation of the hydrogenase into an oxygen stable form. Components present in the growth medium playing such a role could be iron and sulfide. It seems possible that they could reconstitute oxygen-damaged iron–sulfur clusters.

Abbreviations: EDTA, ethylene diaminetetraacetate; DCIP, 2,6-dichlorophenol-indophenol

Here, we report a method to re-oxidize the hydrogen-reduced hydrogenase of *D. vulgaris*, which converts this enzyme back into its oxygen-stable form.

2. MATERIALS AND METHODS

2.1. *Purification of enzymes*

Hydrogenase of *D. vulgaris* strain Hildenborough was purified as in [1]. Cytochrome c_3 of this bacterium was purified from a side fraction of the hydrogenase purification as in [5]. The purity index of this cytochrome, defined as $(A^{red}_{553}-A^{red}_{570})/A^{ox}_{280}$, was 0.31.

2.2. *Hydrogenase activity measurements*

The hydrogen production activity was determined manometrically, the conditions were those in [3]. The hydrogen oxidation activity was determined spectrophotometrically as in [2], in 50 mM Tris (pH 8.0) and 1 mM methyl viologen at 30°C. 2-Mercaptoethanol and a derivative of vitamin B_{12} was used as an oxygen scavenging system. The hydrogen consumption activity was recorded on an Aminco DW-2a spectrophotometer equipped with an Aminco Midan T microprocessor to synchronize the hydrogen consumption activity, as

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

reflected by the formation of methyl viologen semiquinone, determined at 604 nm, and the derivative of the activity curve. It appeared that in case of the oxidized enzyme a sigmoidal type of activity curve was observed. By recording the derivative at each point of the activity curve the hydrogenase activity could be correlated with the redox potential of the methyl viologen redox couple. Under the above conditions the activity was maximal at a redox potential of −414 mV (± 2 mV). No sigmoidal type of activity curve was observed for the reduced enzyme, which makes this type of hydrogenase assay a method to discriminate between the oxidized and reduced form of the *D. vulgaris* hydrogenase. About this difference in activity curve between the oxidized and reduced hydrogenase we will report later.

2.3. *Oxidation of hydrogen reduced hydrogenase*

A 6 ml glass bottle, equipped with a suba-seal stopper, contained in 1 ml final vol.: 0.5 μM *D. vulgaris* hydrogenase; 0.5 mg bovine serum albumin; 10 mM phosphate buffer (pH 7). This bottle was made anaerobic by evacuating and filling with argon gas. If not stated otherwise this solution also contained 0.1 mM ferrous iron (Mohr's salt), 0.1 mM sodium sulfide, and 0.2 mM EDTA (pH 7), which will be denoted as the iron, sulfide, EDTA mixture. The concentrations of iron and EDTA are 4-times the concentration of these salts in the growth medium of the bacterium [6].

The iron, sulfide, EDTA mixture was added from an anaerobic stock solution containing 10 mM, 10 mM and 20 mM of the salts, respectively. EDTA is necessary to prevent precipitation of iron-sulfide. Next argon was replaced by hydrogen gas to reduce the hydrogenase. After about 20 min it was checked by the hydrogen uptake assay whether the hydrogenase was reduced. Next, the hydrogen gas was carefully removed by evacuating and filling with argon gas (at least 5 cycles) and an anaerobic solution (under argon gas) of oxidant was added. The oxidants used were cytochrome c_3 from *D. vulgaris* Hildenborough (4 × n = 1; averaged sum of the midpoint potentials is −340 mV [7], horse heart cytochrome c (n = 1; E_m = +250 mV) and DCIP (n = 2; $E_{m,7}$ = +220 mV). The final concentrations of the oxidants used were 0.25 mM, 0.3 mM and 0.15 mM, respectively. If not stated otherwise, an anaerobic solution of Tris–EDTA (pH 9) was next added (final concentrations of both 50 mM) and the suba-seal stopper removed. If necessary part of this sample was dialyzed against 50 mM Tris (pH 8.0); the other part was used to determine the inactivation of the hydrogenase in time.

All gases used had been passed over a BASF column at 120°C and led through a solution of photoreduced methyl viologen in order to remove trace amounts of oxygen; all solutions were kept at 4°C.

3. RESULTS AND DISCUSSION

Fig.1 shows the effect of addition of oxidant to the oxidized hydrogenase in the absence (fig.1A) and presence of the iron, sulfide, EDTA mixture (fig.1B). In both cases addition of cytochrome c immediately inactivated the hydrogenase by 90%. In the absence of the iron, sulfide, EDTA mixture the half-life upon addition of cytochrome c_3 or DCIP is about 3 days. Addition of the iron, sulfide, EDTA mixture diminishes the half-life in the presence of cytochrome c_3 to about 1 day, but strongly increases the half-life in the presence of DCIP. The decrease in activity of the hydrogenase in the absence or presence of the iron, sulfide,

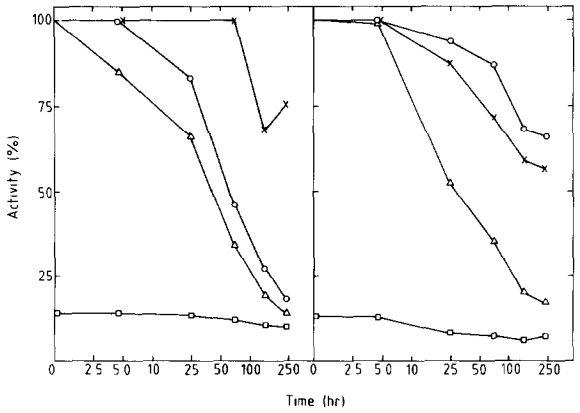

Fig.1. Effect of addition of oxidant on the inactivation of the oxidized hydrogenase in the absence (A) and presence (B) of the iron, sulfide, EDTA mixture. The oxidants used were: (○—○) DCIP; (△—△) cytochrome c_3; (□—□) cytochrome c; (×—×) O_2 (air). At t = 0 the activities were determined in the hydrogen production assay after addition of the oxidant and expressed as percentage activity of the untreated enzyme.

EDTA mixture, after 10 days was in both cases about 30%. The difference in stability of the oxidized enzyme in the presence of DCIP or cytochrome *c* is striking, since both species have about the same midpoint potentials. Recently it has been observed that ferricyanide (E_m = +440 mV) also effectively inactivates the enzyme (H.J. Grande, submitted). It is noticeable that both cytochrome *c* and ferricyanide are $n = 1$, and DCIP is an $n = 2$ oxidant, which might explain the difference in response of hydrogenase towards these oxidants. In fig.2, the effect of oxidation of the hydrogen reduced hydrogenase in the absence (fig.2A) and presence (fig.2B) of the iron, sulfide, EDTA mixture is shown. With exception of cytochrome *c*, the presence of the latter mixture increases the half-life time after re-oxidation from about 10 min to 2 h. In both cases cytochrome *c* exhibits again a detrimental effect on the hydrogenase and no further experiments were performed with this oxidant.

After re-oxidation none of the preparations showed in the hydrogen uptake assay the sigmoidal type of activity curve, with a maximum activity reached at −414 mV, characteristic for the enzyme in its oxygen-stable oxidized state. This also indicates that the enzyme had not been converted into its original oxygen-stable state.

Washing with Tris–EDTA (pH 9) is essential to extract the hydrogenase of *D. vulgaris* [1]. Under physiological conditions the hydrogenase produces hydrogen and will be in its reduced, thus oxygen-sensitive state. This suggests that Tris–EDTA (pH 9) in combination with an(other), yet unknown factor(s), probably oxidant(s), converts the enzyme from an oxygen-sensitive into an oxygen-insensitive form. Therefore, we determined the effect of addition of Tris–EDTA (pH 9) on the oxygen stability of the reduced enzyme and of the re-oxidized enzyme. This effect of addition of Tris–EDTA (pH 9) on the oxygen stability of the reduced or re-oxidized enzyme in the absence (fig.3A) or presence (fig.3B) of the iron, sulfide, EDTA mixture is shown. It is clear that re-oxidation with DCIP in the presence of the iron, sulfide, EDTA mixture, followed by addition of Tris–EDTA (pH 9) results in the conversion of the reduced, oxygen-sensitive hydrogenase into an oxygen-insensitive enzyme. Dialysis of this preparation for 24 h against 50 mM Tris (pH 8) did not lead to any decline in activity. After addition of DCIP then Tris–EDTA, the hydrogenase

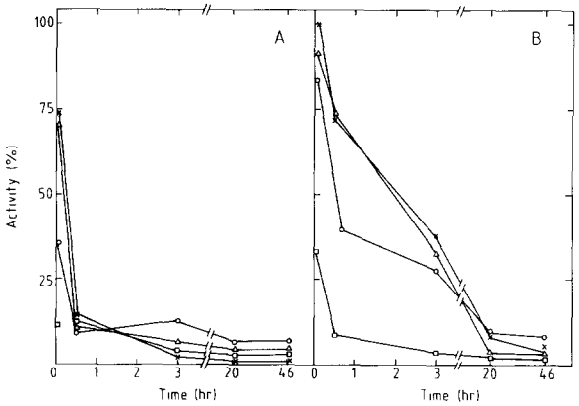

Fig.2. Effect of addition of oxidant on the inactivation of the hydrogen reduced hydrogenase in the absence (A) and presene (B) of the iron, sulfide, EDTA mixture. The oxidants used were: (○—○) DCIP; (△—△) cytochrome c_3; (□—□) cytochrome *c*; (×—×) O_2 (air). At $t = 0$ the activities were determined in the hydrogen production assay and expressed as percentage activity of the untreated enzyme after addition of the oxidant (DCIP, cytochrome c_3), before removing the suba-seal stoppers.

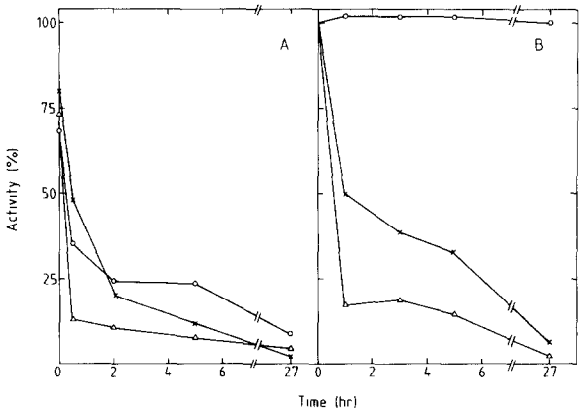

Fig.3. Effect of addition of oxidant and Tris–EDTA (pH 9) on the inactivation of the hydrogen-reduced hydrogenase in the absence (A) and presence (B) of the iron, sulfide, EDTA mixture. The oxidants used were: (○—○) DCIP; (△—△) cytochrome c_3; (×—×) O_2 (air). At $t = 0$ the activities were determined in the hydrogen production assay and expressed as percentage activity of the untreated enzyme after addition of the oxidant (DCIP, cytochrome c_3) and Tris–EDTA (pH 9) before removing the suba-seal stoppers.

showed the hydrogen oxidation activity curve characteristic for the oxidized enzyme; a sigmoidal type of activity curve with a maximum activity at a potential of −414 mV.

Omission of the iron, sulfide, EDTA mixture or oxidation with either cytochrome c_3 or oxygen is detrimental to the oxygen stability of the hydrogenase. In all these latter cases, none of the preparations showed, after addition of Tris–EDTA (pH 9) the oxidized type of activity curve in the hydrogen oxidation assay. Oxygenation of the cells in the growth medium before carrying out the extraction resulted [6] in a higher yield of hydrogenase as compared with the absence of this aeration procedure. In our experiments the use of O_2 as oxidant resulted, under all conditions described, in a rapid loss of activity.

Since re-oxidation of the reduced hydrogenase with DCIP in the presence of the iron, sulfide, EDTA mixture, followed by addition of Tris–EDTA (pH 9) leads to the oxygen-stable state of the enzyme, this oxidation was performed with the individual components of the mixture. It was found that neither EDTA, iron nor sulfide but only the combination iron, sulfide, EDTA as well as the combination iron, EDTA, results in the conversion into an oxygen-stable enzyme upon oxidation with DCIP followed by addition of Tris–EDTA (pH 9) showing that sulfide is not essential. Hydrogen oxidation activity of the oxidized or re-oxidized samples was lower in all cases by a factor 2.4 ± 0.2, as compared to the activity of the samples when they were (re)reduced by hydrogen. Furthermore, the inactivation patterns as shown in fig.1–3 are the same as determined in the hydrogen production as in the consumption assay.

Upon reduction the oxygen-stable state of the enzyme converts into an oxygen-sensitive state. As is suggested by the non-linearity of the hydrogen oxidation activity curve with methyl viologen as electron acceptor the oxidized, oxygen-stable form is, in a time-dependent process, irreversibly converted into an oxygen-sensitive form. (The sigmoidal type of hydrogen uptake activity curve was also observed for the oxidized hydrogenase with the physiological electron acceptor cytochrome c_3; J.W. van Leeuwen, unpublished.) We suggest that this reduction is probably associated with a conformational change of the enzyme at the level of (a) the iron-sulfur cluster(s) of the enzyme with (a) cluster(s) irreversibly reduced. Due to this irreversible type of reduction the enzyme becomes oxygen-sensitive. Only under special conditions, such as described above, this oxygen-sensitive state is converted back into its oxygen-insensitive state. Since only the $n = 2$ oxidant DCIP and not the $n = 1$ oxidant cytochrome c, both having about the same midpoint potential, is able to establish this conversion, it seems that a 'super'-reduced cluster is irreversibly oxidized in a $n = 2$ step into its 'super'-oxidized form. However, it should be kept in mind that both iron, EDTA plus Tris–EDTA (pH 9) are essential for the re-oxidation process. We have reported [7] that the midpoint potential of an iron, EDTA complex is +50 mV. The iron–EDTA complex might thus act synergistically in combination with DCIP.

Recently it has been reported that cytochrome c_3 could be used to obtain an oxygen stable reoxidized hydrogenase [10]. However, these experiments clearly show that this could not be reproduced. The reason for this discrepancy is not known. Similar experiments performed with the hydrogenase of *M. elsdenii* did not result in an oxygen-stable hydrogenase indicating that the oxygen-sensitivity of this enzyme is an intrinsic property.

ACKNOWLEDGEMENTS

The authors wish to thank Miss C.M. Verstege for typing the manuscript and Mr M.M. Bouwmans for drawing the figures. This work was supported by a grant of the EC under contract ESD-029-NL and by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for the Advancement of Pure Research (ZWO).

REFERENCES

[1] Van der Westen, H.M., Mayhew, S.G. and Veeger, C. (1978) FEBS Lett. 86, 122–126.
[2] Van Dijk, C., Mayhew, S.G., Grande, H.J. and Veeger, C. (1979) Eur. J. Biochem. 102, 317–330.
[3] Chen, J.S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta 371, 283–298.

[4] Mayhew, S.G., Van Dijk, C. and Van der Westen, H.M. (1978) in: Hydrogenases: Their Catalytic Activity, Structure and Function (Schlegel, H.G. and Schneider, K. eds) pp.125–140, Golze, Göttingen.
[5] Van Leeuwen, J.W., Van Dijk, C., Grande, H.J. and Veeger, C. (1982) Eur. J. Biochem. 127, 631–637.
[6] Van der Westen, H.M., Mayhew, S.G. and Veeger, C. (1980) FEMS Microbiol. Lett. 7, 35–39.
[7] Van Dijk, C., Van Leeuwen, J.W., Veeger, C., Schreurs, J.P.G.M. and Barendrecht, E. (1982) Bioelectrochem. Bioenerg. 9, 743–759.
[8] Van Dijk, C., Grande, H.J., Mayhew, S.G. and Veeger, C. (1980) Eur. J. Biochem. 107, 251–261.
[9] Grande, H.J., Van Berkel-Arts, A., Bregh, J., Van Dijk, C. and Veeger, C. (1983) Eur. J. Biochem. 131, 81–88.
[10] Van Dijk, C., Grande, H.J. and Veeger, C. (1981) in: Photochemical, Photoelectrochemical and Photobiological Processes (Hall, D.O. and Palz, W. eds) vol.1, pp.190–194, Reidel, Dordrecht, Boston MA.