Effects of thiols and mercurials on the periplasmic hydrogenase from *Desulfovibrio vulgaris* (Hildenborough)

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The H_2 -oxidation, H_2 -production and H - 3H -exchange activities of the periplasmic hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) were almost completely abolished by $Hg(II)$ and the organic mercurials *p*-chloromercuribenzoate (pCMB) and *p*-hydroxymercuriphenylsulphonate. The thiol-modifying reagents *N*-ethylmaleimide, iodoacetate, dithionitrobenzoate and 2-nitro-5-thiocyanobenzoate had no effect on the activities. Kinetic and spectroscopic measurements suggest that inactivation by pCMB involves at least two reactions; a rapid reaction that is reversed by thiols, and a second, slower and irreversible

reaction that occurs at high concentrations of the mercurial. The irreversible reaction was associated with loss of visible absorbance, indicative of a disrupted iron–sulphur cluster(s). The effects on the H - 3H -exchange activity indicate that the reversible modification affects the H_2 -activating site. Enzyme that had lost activity due to pCMB treatment, or during long-term storage, was reactivated by thiols. This reactivation was followed by a slower irreversible inactivation, as also occurred with native enzyme; the inactivation was O_2 dependent and it was partly prevented by catalase, suggesting that H_2O_2 may be involved.

INTRODUCTION

Hydrogenases (EC 1.18.99.1 and EC 1.12.2.1) catalyse the reversible oxidation of H_2 in the presence of a suitable electron carrier. They have been isolated from a variety of micro-organisms, and their structures and catalytic properties have been found to vary widely [1–4]. The enzyme isolated from the periplasm of *Desulfovibrio vulgaris* (Hildenborough) uses cytochrome c_3 as its physiological electron carrier [5], and it is highly active both in the oxidation of H_2 and in the reduction of protons to form H_2 [6]. It consists of two subunits of molecular mass 46 and 10 kDa [7–10], and it contains up to 16 atoms of iron and 14 atoms of acid-labile sulphur [9] arranged in two $[4Fe-4S]$ clusters and (an) additional uncharacterized cluster(s). It lacks nickel and therefore it differs from most other hydrogenases that have been isolated. The gene for the enzyme has been cloned and sequenced, and expressed in *Escherichia coli*, although in an inactive form [7,11,12].

Most studies on the structure and function of the enzyme have focused on the spectroscopic properties associated with the iron–sulphur centres [13–15], and very little is known about the chemical reactivity of the enzyme. Sadana and Rittenberg [16] reported that a crude preparation is inactivated by mercurials and other thiol-modifying agents such as *N*-ethylmaleimide and iodoacetate. We have now observed that a highly purified preparation of the enzyme is similarly inactivated by mercurials, but that it is unaffected by a variety of other thiol reagents. The inactivation by mercurials is reversed by thiol-containing compounds. During the course of this work it was found that thiols also reactivate enzyme which has become inactivated during long-term storage, but that under some conditions treatment with thiol leads to irreversible inactivation. A preliminary account of parts of this work has appeared [17].

METHODS AND MATERIALS

Bacterial growth and purification of hydrogenase

D. vulgaris (Hildenborough) NCIB 8303 was grown anaerobically

in Saunders medium [18], modified to contain EDTA [6]. A crude preparation of periplasmic proteins including hydrogenase was made immediately after harvesting [19], and hydrogenase was purified at 4 °C by a method based on that described previously [6]. An additional chromatographic step on DEAE-cellulose was used in a final step to give a homogeneous preparation. The purified enzyme showed two bands on SDS/PAGE corresponding to molecular masses of 40 and 10.5 kDa. The specific activity was 3600–3780 units/mg as judged by manometric H_2 production using 1 mM methyl viologen as electron carrier [6], and assaying protein according to Lowry et al. [20].

Assays for hydrogenase

Spectrophotometric assays for H_2 oxidation were carried out anaerobically at 30 °C in a glass cuvette, fitted with a Suba-seal stopper [21]. The cuvette contained, in a final volume of 3 ml: 200 μ mol of Tris/HCl buffer (pH 8), 1.5 mg of BSA, 3 μ mol of benzyl viologen and 30 nmol of sodium dithionite. Buffer and viologen were made anaerobic in the cuvette and equilibrated with H_2 gas. An aerobic solution of BSA (10 μ l) was added, followed by sodium dithionite (3 μ l) to remove the last traces of O_2 . The reaction was started by addition of hydrogenase (3–20 μ l). The reaction accelerated [21], and measurements of the reaction rate were therefore made 20 min after the addition of enzyme. At this time, the rate was directly proportional to the enzyme concentration.

Manometric assays of H_2 production were carried out in Warburg manometers at 30 °C [6,22]; they contained in the main compartment, in a final volume of 2 ml: 100 μ mol of Tris/HCl buffer (pH 8), 2 μ mol of methyl viologen, 30 μ mol of sodium dithionite, and 1 mg of BSA. The centre well contained 0.2 ml of 10% (w/v) NaOH. The side arm, in a volume of 0.1 ml, contained: hydrogenase (to produce up to 1 μ mol of H_2 /min); 10 μ mol of Tris/HCl buffer (pH 8) and 0.05 mg of BSA. The gas phase was N_2 . The reaction was started by tipping the contents of the side arm into the main compartment; H_2 production was linear with time.

Abbreviations used: pCMB, *p*-chloromercuribenzoate; DTT, dithiothreitol.

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Isotope-exchange activity was determined by measuring the incorporation of ^3H from $^3\text{H}_2$ into water [23].

Long-term anaerobic incubation of hydrogenase

Experiments that required periodic sampling of hydrogenase with maintenance of anaerobic conditions for several days were carried out in test tubes that were fitted with a Suba-Seal stopper and which had two compartments. Compartment A contained the incubation mixture of hydrogenase and dithiothreitol (DTT); compartment B contained an O_2 -scavenging mixture of reduced methyl viologen in Tris/HCl buffer. Methyl viologen (1 mM) and Tris/HCl buffer, pH 8, (90 mM) in 0.4 ml were first made anaerobic in compartment B by evacuation and filling the test tube with N_2 via a syringe needle inserted through the stopper. Sodium dithionite (0.2 μmol) was then added to reduce the methyl viologen. Hydrogenase and DTT, both made anoxic with N_2 , were added to compartment A. Additions were made while maintaining a flow of N_2 through the tube. Samples were withdrawn periodically for analysis of H_2 -oxidation activity. Further additions of sodium dithionite were made at intervals to compartment B to maintain methyl viologen in its reduced form.

Chemical methods

Protein concentration was estimated [20] using BSA as standard; hydrogenase determined by this method was first precipitated with 5% (w/v) trichloroacetic acid and then redissolved in 0.1 M NaOH.

Materials

N_2 and H_2 were purified by passing them over BASF catalyst (R3-11) heated to 110 $^\circ\text{C}$, and then through a solution of reduced methyl viologen, which served to remove the last traces of O_2 , to give a visible indication that the system was anoxic, and to saturate the gas with water vapour. Methyl- and benzyl viologen, BSA (A7638), catalase, DTT and 2-mercaptoethanol were obtained from Sigma.

RESULTS AND DISCUSSION

Effects of mercurials on catalytic activity

In contrast with an earlier study which found that a crude preparation of hydrogenase from *D. vulgaris* was inactivated by *N*-ethylmaleimide and iodoacetate [16], we observed no effect of these two reagents on the activity of the purified periplasmic enzyme, and dithionitrobenzoate and 2-nitro-5-thiocyanobenzoate were similarly without effect [17]. However, the enzyme was inactivated by HgCl_2 and by the organic mercurials *p*-chloromercuribenzoate (pCMB) and *p*-hydroxymercuriphenylsulphonate. The rate of inactivation by $\text{Hg}(\text{II})$ (Figure 1a) was higher than with the organic mercurials [Figures 1(b) and 1(c)]. Although the rates varied with the concentration of each reagent, plots of $\log_{10}$ of the activity remaining versus time, when the reagents were present in large excess over the enzyme, were not linear, indicating that the reactions did not obey pseudo-first-order kinetics (Figure 1, inserts). In the case of the reaction with pCMB, which was allowed to proceed for up to 30 h, there was an indication of an initial relatively rapid loss of activity followed by a slower phase during which the residual activity declined over many hours (Figure 1c); however, even the slow phase did not seem to follow first-order kinetics.

It is noteworthy that prolonged incubation with pCMB did not completely inactivate the enzyme. Even after treatment with a high concentration of the mercurial for nearly 30 h, a residual activity of 1–2% remained. The residual activity may have been due to a small fraction of non-reacting molecules in the preparation, perhaps associated with different enzyme forms as separated recently by isoelectric focusing [24]. Alternatively, the residual activity might reflect the equilibrium constant for the mercaptide formed with the enzyme, although values reported for mercaptides (approx. 1×10^{-20} M [25]) do not seem compatible with the activities observed. The most likely explanation

Figure 1 Inactivation of hydrogenase by mercurials

Hydrogenase was incubated at 4 $^\circ\text{C}$ with the mercurial compound indicated. Samples were taken at intervals and assayed for H_2 -oxidation activity. The activity is expressed as a percentage of the activity of untreated enzyme. The inserts show plots of $\log_{10}$ [activity remaining (%)] versus time. The incubation mixtures contained: 0.1 M Tris/HCl buffer, pH 8; BSA, 0.25 mg/ml; hydrogenase and the mercurial as indicated. (a) 542 ng of hydrogenase and 10 μM HgCl_2 in 0.12 ml; (b) 9 ng of hydrogenase and 0.1 mM ($\circ$, $\bullet$) or 1 mM ($\square$, $\blacksquare$) *p*-hydroxymercuriphenylsulphonate in 0.06 ml; (c) 17 ng of hydrogenase and 11 μM ($\square$, $\blacksquare$) or 110 μM pCMB ($\circ$, $\bullet$) in 0.1 ml. Open and closed symbols refer to duplicate experiments.

Figure 2 Effect of time of incubation with pCMB on the reactivation of hydrogenase by cysteine

Hydrogenase was first incubated at 4 °C with pCMB. The incubation mixture contained (in 0.2 ml): pCMB; Tris/HCl, pH 8, 0.1 M; BSA, 0.25 mg/ml; hydrogenase, 34 ng. At the times indicated, samples were withdrawn and mixed at 4 °C with an equal volume of cysteine in 0.1 M Tris/HCl buffer, pH 8, to give a final cysteine concentration of 2 mM. Samples from the second incubations were assayed at intervals for H_2 -oxidation activity. The activity of the samples (percentage of the activity before treatment with pCMB) is plotted versus the time of incubation with cysteine. (a) pCMB (1.1 mM), incubated for 0.5 (●), 2 (■), 4 (□) and 8 h (○). (b) pCMB (10 μM), incubated for 0 (■), 3 (□), 24 (▲), and 672 h (●); note that this experiment included a control incubation without pCMB (data for 0 h incubation).

is that pCMB-reacted molecules retain catalytic activity. It is interesting that the residual activity of pCMB-inactivated hydrogenase from *D. vulgaris* is similar to the normal activities of the nickel- and iron-containing (Ni-Fe) hydrogenases [2].

The effects of mercurials have been studied with certain other hydrogenases. The Ni-Fe hydrogenases from *E. coli* and *Chromatium vinosum* and the Fe hydrogenase from *Megasphaera elsdenii* are not affected by pCMB [26,27]. The enzyme from *Nocardia opaca* 1b is inactivated by pCMB, and the enzyme becomes more susceptible when it is incubated with H_2 and NAD^+ , suggesting that pCMB may react at the H_2 -activating site [28]. The H - 3H -exchange assay for hydrogenase does not involve electron transfer, and therefore it can be used to test for integrity of the H_2 -activating site [1,23]. When *D. vulgaris* hydrogenase was incubated with 1 mM pCMB and periodically assayed, the H - 3H -exchange and H_2 -oxidation activities decreased in parallel,

Figure 3 Effect of DTT on the activity of hydrogenase inactivated during storage

Hydrogenase that had become 82% inactivated during storage at -20 °C was incubated with DTT at 4 °C in air (▲) or under N_2 (●, ■). Samples were removed at intervals and assayed for H_2 -oxidation activity. The incubations contained, in 0.16 ml: 0.05 M Tris/HCl, pH 8, 0.25 mg/ml BSA, 8.5 mM DTT and 23 ng of hydrogenase. The activity, expressed as a percentage of the activity before storage, is plotted versus the time of incubation. The activity of a control incubation under N_2 without addition of DTT is also shown (■).

suggesting that the mercurial reagent directly affects the active site (results not shown).

It is interesting that although pre-incubation of enzyme with pCMB led to inactivation in all three assay systems used, the inclusion of pCMB (1 mM) in the manometric assay for H_2 production was without effect. The enzyme remained fully active for at least 20 min.

Reactivation by thiols

pCMB-inactivated enzyme

The catalytic activity of hydrogenase that had been inactivated with pCMB was restored by addition of a thiol-containing reagent. The extent of reactivation depended on the concentration of mercurial used to inactivate the enzyme, the time of pre-incubation of enzyme with mercurial, the nature of the reactivating thiol, and the time of incubation of the thiol with the enzyme. Initial experiments suggested that the recovery of activity was greater with cysteine than with 2-mercaptoethanol or DTT. Therefore most studies on the reactivation of pCMB-treated enzyme were done with cysteine. When cysteine was added to enzyme, that had been 98% inactivated by pCMB for 30 min, the activity was almost completely recovered, but it then declined slowly during a further 4 h of incubation (Figure 2a). The maximum activity that was recovered decreased with increasing times of pre-treatment with pCMB until, after 8 h, only 8% of the original activity was recovered by addition of cysteine. This experiment confirms that pCMB inactivation occurs in two phases; a relatively rapid loss of most of the activity in a reaction that is reversed by cysteine, followed by a much slower reaction that is not reversed by the thiol. The recovery of activity in the H_2 -oxidation assay occurred in parallel with the recovery of activity in the H - 3H -exchange assay (results not shown), again suggesting that the reversible inactivation by pCMB affects the

H₂-activating site rather than only a component of the electron transfer pathway to and from the active site.

Hydrogenase from *D. vulgaris* is relatively stable in air [6], but it is rapidly and irreversibly inactivated by O₂ after reduction with H₂ or dithionite ion [5]. The possibility that a high concentration of pCMB (1 mM) induces a slow structural change that causes the enzyme to become O₂-sensitive was ruled out by carrying out parallel experiments in air and under N₂. The fraction of activity that could not be recovered by subsequent incubation with cysteine was the same, indicating that the irreversible loss was not caused by O₂.

The irreversible reaction seems to occur only at high concentrations of pCMB. When cysteine was added to enzyme that had been incubated with 10 μ M pCMB for 3 h, full activity was restored (Figure 2b). A similar recovery occurred after 24 h of pCMB treatment although the enzyme had been almost fully inactivated. After 28 days, only 66% recovery occurred, but a control experiment showed that untreated enzyme also lost activity during this period (55% loss), and that this activity was not recovered by addition of cysteine.

Enzyme inactivated during storage

A variable loss of activity occurs when hydrogenase from *D. vulgaris* is stored at -20 °C. We have observed that such partially inactive enzyme can be reactivated by addition of a thiol. One preparation that had been stored for 2 years retained only 18% of its activity. Up to 54% of the original activity of this preparation was restored by treating the partially inactive enzyme with DTT. The extent of reactivation depended on the time and temperature of the incubation and on the presence of O₂. When enzyme was treated with DTT in air, the activity of the enzyme increased transiently (to 28%) but it then decreased again during the following 10 days (Figure 3). The activation was much greater and more prolonged when the treatment was carried out under N₂, and although the activity subsequently declined, its rate of loss was low by comparison with the aerobic incubation. Precautions were taken during sampling in the anaerobic experiment to protect the enzyme from air, but the possibility of O₂ causing the slow inactivation in this experiment cannot be ruled out. The anaerobic addition of DTT had no effect on freshly prepared and fully active enzyme; the enzyme was neither activated nor inactivated. In contrast, aerobic treatment of the enzyme caused inactivation similar to the control level of Figure 2(B) for cysteine.

It seemed possible that the inactivation under aerobic conditions caused by DTT (and also by cysteine) could be due to the reduction of O₂ to H₂O₂ and the subsequent reaction of H₂O₂ with a sensitive group in the enzyme. Such reactions have been noted with rhodanese [29] and phenylalanine hydroxylase [30]. However, catalase (1 mg/ml) was found to only partially protect hydrogenase against the inactivating effects of DTT; 61% and 86% of the initial activity were lost during 21 h incubation at 30 °C, pH 8, in the presence and absence respectively of catalase.

Effects of pCMB and cysteine on the visible absorbance of hydrogenase

Mercurial compounds are known to react not only with thiols but also with Fe-S clusters; for example, the addition of sodium mersalyl to the 8Fe-8S ferredoxin from *Clostridium pasteurianum* leads to a bleaching of the visible absorbance [31]. When hydrogenase was reacted with pCMB the visible absorbance decreased (Figure 4). A plot of log₁₀ ΔA_{400} versus time was non-linear, again ruling out a pseudo-first-order reaction. The absor-

Figure 4 Effect of pCMB on the absorbance spectrum of hydrogenase

Hydrogenase and pCMB were mixed and the absorbance changes monitored at 400 nm (insert); spectra (top to bottom) were recorded at 0, 0.5, 1.5 and 3.5 h. The spectrophotometer cuvette contained in 0.8 ml at 10 °C: 1 mM pCMB; 0.058 M Tris/HCl, pH 8, and 0.12 mg of hydrogenase.

bance of hydrogenase in the visible region is attributable to the Fe-S clusters in the enzyme, and the observed losses suggest that at least one cluster is disrupted by pCMB. Enzyme assay showed that the partial loss of visible absorbance was accompanied by almost complete loss of both H-³H-exchange and H₂-oxidation activities (95% and 97% loss for the exchange and oxidation assays respectively).

Treatment of the partially bleached enzyme with 5 mM cysteine led to a small initial increase in absorbance which decreased to its original value during incubation for 1.5 h. At the same time the catalytic activities in the H₂-oxidation and H-³H-exchange assays increased to 30% and 38% respectively of the activity of the native enzyme. These observations suggest that the reactivation of pCMB-inactivated enzyme is not due to reconstitution of (an) Fe-S cluster(s).

The most likely explanation of the effects of pCMB on the enzyme activities and u.v.-visible absorbance, and for the effect of cysteine on the pCMB-inactivated enzyme is that, first, a low concentration of pCMB (10 μ M) causes inactivation of the enzyme without affecting the Fe-S clusters. In this case the enzyme can be fully reactivated by cysteine (see Figure 2b). Secondly, that a higher concentration of pCMB (1 mM) causes an additional but slower reaction in which the Fe-S clusters are destroyed; the slower reaction is not reversed by added thiols.

Since the reversible reaction does not lead to changes in the u.v.-visible spectrum, it is unlikely that it involves acid-labile sulphur or those cysteinyl sulphur atoms involved in the Fe-S clusters. The enzyme contains a total of 18 cysteine residues [8,10], eight of which are required to bind two ferredoxin-like [4Fe-4S] clusters. As discussed by Meyer and Gagnon [32], some of the remaining cysteine residues almost certainly serve to bind

the remaining 2–6 iron atoms. However, this still leaves a number of residues which may be present as cysteine rather than cystine and which could react with pCMB. One or more of these residues is the most likely site for the reversible reaction. If this is the case, it is possible that a sulphhydryl group is necessary for activity. Such a thiol could act as the weak acid or weak base required in several schemes proposed for the mechanism of action of hydrogenase [1].

Hydrogenase from *D. vulgaris* shows an induction period in the H_2 -oxidation assay during which the reaction accelerates as the enzyme becomes activated [21]. This induction period is not observed in the standard manometric assay for H_2 production [6] in which it is thought that the enzyme is rapidly converted by the strong reductants sodium dithionite and methyl viologen from 'resting' enzyme to the 'active' state [1,24]. We can now propose that a third form of the enzyme, an 'inactive' state, is formed during long-term storage at -20°C . DTT converts this 'inactive' enzyme only as far as the 'resting' state because the resulting enzyme still shows the induction period in the H_2 -oxidation assay. Three states with different catalytic and spectroscopic properties have previously been observed with other hydrogenases, such as those from *Desulfovibrio gigas* [33] and *Ch. vinosum* [34]. However, these enzymes contain nickel, and interconversions between their three states are associated with changes in the redox and/or co-ordination state of this metal [33,34].

Supported by EOLAS (NBST grant SRG 5/82) and the Irish Department of Education.

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