THE EFFECT OF OXYGEN SPECIES ON THE ENZYMATIC ACTIVITY OF HYDROGENASE

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1. Introduction

Hydrogenase, which catalyses the reversible activation of H_2, is inactivated upon exposure to oxygen. Although the extent of this inactivation depends on the source of the enzyme [1–3] relatively little information is available concerning the processes involved. The purification and characterisation of these enzymes have been often hampered by their oxygen sensitivity. Moreover, in view of the present application of hydrogenase to biological solar energy conversion systems to produce H_2 [4,5], it was considered important to determine whether the in vitro inactivation of hydrogenase is attributable to oxygen per se or to products of its metabolism.

Numerous studies have indicated that oxygen toxicity may, in part, be associated with the production of the superoxide anion radical ($O_2^{\cdot-}$) [6,7]. In addition there is some evidence that the more highly reactive hydroxyl radical ($^{\cdot}OH$) [8] and/or singlet oxygen ($^1\Delta O_2$) [9] may be involved.

We report here the effects of various oxygen species upon the enzymatic activity of hydrogenases isolated from two microbial sources. The results suggest that:

(i) Molecular oxygen per se is directly involved in the rate limiting step of oxygen inactivation;
(ii) The inactivation by $O_2^{\cdot-}$ and O_2 occur by different mechanisms which appear to be directed at different sites of the enzyme.

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2. Materials and methods

Escherichia coli hydrogenase was purified to near homogeneity [10]. *Clostridium pasteurianum* hydrogenase was used as a partially purified enzyme [11]. Superoxide dismutase (Mn-containing form) was purified from *Neurospora crassa* [12] and had an activity of 2000 units/mg; one unit of activity inhibited the rate of nitro blue tetrazolium reduction by 50% under the conditions in [13]. Hydrogenase activity was assayed by the rate of H_2 evolution with 10 mM sodium dithionite and 2.5 mM methyl viologen at 30°C [14]; one unit of hydrogenase activity catalysed the evolution of 1 μmol H_2/min.

Effect of various incubation conditions upon hydrogenase activity: The various reagents were added to the hydrogenase (*E. coli*, 2 units, or *C. pasteurianum*, 15 units) separately or in combination to give a final reaction volume of 2 ml. The mixture was incubated with shaking (250 rev./min) at 20°C under an atmosphere of O_2, air or N_2. At intervals, aliquots (20–100 μl) were withdrawn and the residual hydrogenase activity determined, which is expressed as a percentage of the zero time control. Periodically the gas phase in the incubation vials was checked for O_2 content by gas chromatography. Control vials containing either no hydrogenase or boiled hydrogenase samples were included over prolonged periods of incubation. These showed that the measured hydrogenase activity was not due to bacterial contamination. Superoxide was generated enzymatically by the action of xanthine oxidase on xanthine (under aerobic conditions) or was added directly to the system in the form of potassium superoxide (KO_2) in dry dimethyl sulfoxide (DMSO, [15]). All chemicals used were of the highest purity commercially available.

3. Results and discussion

3.1. *Oxygen-mediated inactivation of hydrogenase*

It may be seen in table 1 that *E. coli* hydrogenase is inactivated upon exposure to oxygen. We further investigated this inactivation and the effects of various additions to the hydrogenase are shown (table 1). Although catalase was found to protect, this action appears to be non-enzymic since cytochrome *c* and bovine serum albumin had similar effects. These results suggest that *E. coli* hydrogenase which is of membrane origin is protected by its association with other proteins [16]. Superoxide dismutase at low concentrations offered little protection. *N*-Ethylmaleimide (NEM, 1 mM) protected the hydrogenase from oxygen which suggests the involvement of sulphydryl groups. However, glutathione (1 mM) and/or ascorbate (1 mM) afforded no protection (not shown).

3.2. *Effect of a variety of reagents during prolonged incubation of hydrogenase under an atmosphere of air or O_2*

We next investigated whether various additions to *E. coli* hydrogenase would afford long-term protection. Separate additions of mannitol, β-carotene and sodium formate, all scavengers of reactive oxygen species, were without effect. However, as shown in table 2, a combination of mannitol and β-carotene gave some protection, as did a combination of catalase and superoxide dismutase. The most effective protection was observed with a combination of benzoate, catalase and β-carotene.

Table 1
Inactivation of *E. coli* hydrogenase by oxygen[a]

Additions	Residual activity (% of t = 0 control)
N_2 atmosphere (no additions)	95
O_2 atmosphere (no additions)	0
Catalase (500 μg)	74
Cytochrome *c* (horse heart, 1 mg)	82
Bovine serum albumin (1 mg)	73
Superoxide dismutase (1 unit)	14
Catalase (500 μg), superoxide dismutase (1 unit)	88
Catalase (500 μg), bovine serum albumin (1 mg)	84
N-Ethylmaleimide (1 mM)	54

[a] The 2 ml reaction mixture, containing the hydrogenase with the various additions as indicated, was incubated for 20 h

Table 2
Effect of various additions on the in vitro inactivation of *E. coli* hydrogenase under an atmosphere of air or oxygen (residual activity, %)[a]

Incubation conditions	Air		Oxygen	
	6 days	14 days	6 days	14 days
No additions	0	–	0	–
Mannitol (50 mM), β-carotene (1 mM)	31	0	35	0
Catalase (500 μg), superoxide dismutase (4 units)	70	2	10	2
Benzoate (100 mM), catalase (500 μg), β-carotene (1 mM)	66	49	72	48
2,2′-Bipyridyl (1 mM)	55	21	48	18
Benzoate (100 mM)	38	6	25	0
Control (N_2 atmosphere instead of air or oxygen)	65	40		

[a] The residual activity is expressed as a percentage of the zero time control (not the N_2 control)

The stabilisation of the hydrogenase by 2,2′-bipyridyl is also shown in table 2. Although the molecular structure of this compound is similar to methyl viologen, it will not mediate electron transfer to the hydrogenase. Benzyl viologen, a hydrophobic redox carrier which will donate/accept electrons to/from *E. coli* hydrogenase also protects the enzyme from oxygen-mediated inactivation [10].

From the data in table 2 it may be seen that most of the reagents gave similar protection in O_2 as in air, and that none of them stabilised the hydrogenase to a significantly greater degree than that obtained under a N_2 atmosphere. This suggests that the protective effects observed are a direct result of preventing inactivation by O_2 rather than preventing protein denaturation.

3.3. *Effect of reactive oxygen species on hydrogenase activity*

The hydrogenases of *E. coli* and *C. pasteurianum* were irreversibly inactivated by $O_2^{\cdot-}$ (whether added directly or generated enzymatically) and the presence of H_2O_2 in the reaction mixture accelerated the rate of inactivation. In accordance, superoxide dismutase

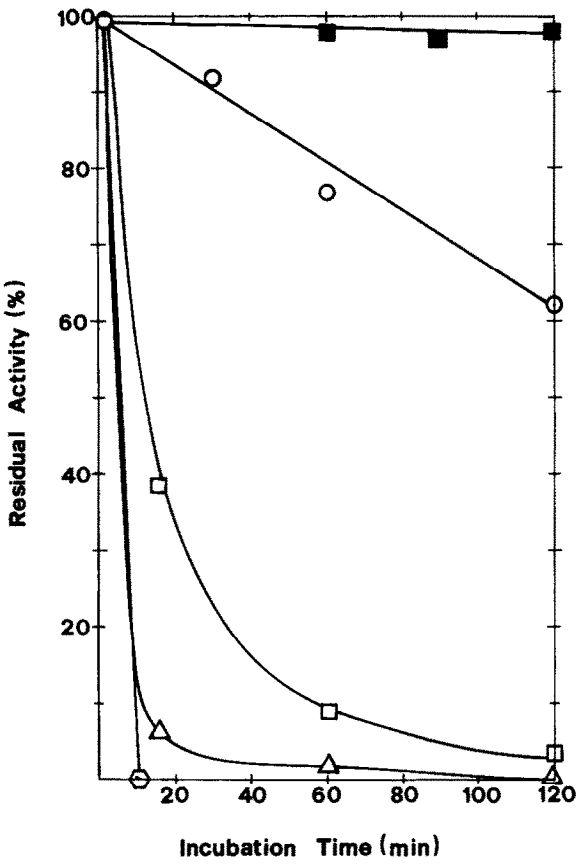

Fig.1. Inactivation of *E. coli* hydrogenase by activated oxygen species and the effect of transition metal ions. The hydrogenase was incubated under an atmosphere of air as in section 2. The residual activity is expressed as a percentage of the zero time control. The 2 ml reaction mixture contained where indicated (X) xanthine, 1 ml saturated solution; (XO) xanthine oxidase, 50 μg; (KO_2) potassium superoxide, 200 μl saturated solution in dry DMSO; H_2O_2, 2 mM; Mn^{2+}, $MnCl_2$, 1 mM; Cu^{2+}, $CuSO_4$, 1 mM. The additions were: (■) no additions; (○) X/XO plus H_2O_2; (△) X/XO plus H_2O_2 plus Cu^{2+}; (◯) KO_2 plus H_2O_2; (□) KO_2 plus H_2O_2 plus Mn^{2+}.

and catalase afforded considerable protection from these reagents (not shown). Fig.1 shows the inactivation of the *E. coli* enzyme together with the effect of two transition elements (Cu^{2+} and Mn^{2+}). Control experiments showed that under the incubation conditions, H_2O_2 did not inhibit the xanthine oxidase. Inactivation of the hydrogenases may have occurred via a mechanism(s) involving one or more oxygen radicals, e.g., $^{\cdot}OH$, $HO_2^{\cdot}$ and $RO^{\cdot}$, and/or metal oxygen complexes, e.g., MO^{2+}. The sensitisation observed in the presence of Cu^{2+} might have occurred by the following mechanism (eq. (1–3)):

$$Cu^{2+} + O_2^{\cdot-} \rightarrow Cu^{+} + O_2 \quad (1)$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\cdot}OH + OH^{-} \quad (2)$$

$$\text{Net: } O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^{-} \quad (3)$$

The protection observed with Mn^{2+} probably resulted from a scavenging of $O_2^{\cdot-}$ [13].

In an attempt to obtain further information on the mechanism(s) of this inactivation by reactive oxygen species, various reagents were added to *E. coli* and *C. pasteurianum* hydrogenases before the addition of superoxide and peroxide. The radical scavengers sodium benzoate (10 mM), sodium formate (10 mM), mannitol (10 mM), butylated hydroxytoluene (10 mM) and α-totopherol (1 mM) did not protect either hydrogenase. Moreoever, the singlet oxygen scavenger β-carotene (10 μM), the anti-oxidant propyl gallate (1–5 mM), the reducing agents glutathione (2 mM) or ascorbate (1–5 mM), NEM (1 mM) and the redox mediator methyl viologen (1–10 mM) nor any tested combination of these reagents afforded any protection.

A variety of compounds will protect hydrogenase from inactivation by O_2 but there appears to be no direct correlation between the types of reagents used and the possible modes of protection. We conclude, however, that molecular O_2 per se is directly involved in the rate-limiting step and that the mechanism of inactivation may not involve direct participation of free superoxide and/or peroxide intermediates. Other oxygen species may be involved since combinations of some scavengers protected the hydrogenase from inactivation by O_2. Although the actual mechanism involved is unclear, it seems that inactivation by O_2 and by $O_2^{\cdot-}$ plus H_2O_2 occur via different mechanisms. It is possible that the inactivation by O_2 occurs via the iron–sulphur active centre since viologen dyes (which interact with the active centre) protect the hydrogenase as also the inhibitor carbon monoxide [17]. We are looking into these possibilities.

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