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Production of superoxide radicals by soluble hydrogenase from *Alcaligenes eutrophus* H16

Klaus SCHNEIDER and Hans G. SCHLEGEL
Institut für Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, D-340 Göttingen, Federal Republic of Germany

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The soluble hydrogenase (hydrogen–NAD^+ oxidoreductase, EC 1.12.1.2) of *Alcaligenes eutrophus* H16 was shown to be stabilized by oxidation with oxygen and ferricyanide as long as electron donors and reducing compounds were absent. The simultaneous presence of H_2, NADH and O_2 in the enzyme solution, however, caused an irreversible inactivation of hydrogenase that was dependent on the O_2 concentration. The half-life periods at 4°C under partial pressures of 0.1, 5, 20 and 50% O_2 were 11, 5, 2.5 and 1.5 h respectively. Evidence has been obtained that hydrogenase produces superoxide free radical anions ($O_2^{-\cdot}$), which were detected by their ability to oxidize hydroxylamine to nitrite. The correlation between O_2 concentration, nitrite formation and inactivation rates and the stabilization of hydrogenase by addition of superoxide dismutase indicated that superoxide radicals are responsible for enzyme inactivation. During short-term activity measurements (NAD^+ reduction, H_2 evolution from NADH), hydrogenase activity was inhibited by O_2 only very slightly. In the presence of 0.7 mM-O_2 an inhibition of about 20% was observed.

Since the first demonstration of the biophotolysis of water by a coupled system of chloroplasts and hydrogenase to produce molecular hydrogen (Benemann *et al.*, 1973), the enzyme hydrogenase has attracted much attention, because it provides a means for the bioconversion of solar energy. One of the main difficulties in the large-scale production of H_2 concerns the O_2 sensitivity of hydrogenase (Rao *et al.*, 1978; Schlegel & Schneider, 1978). In recent years several hydrogenases, particularly those purified from phototrophic bacteria, hydrogen bacteria and *Desulfovibrio* species, have been shown to be stable during storage under air [for a review, see Schlegel & Schneider (1978)]; however, in most cases no information was given on the influence of O_2 on hydrogenase under reaction conditions. Inhibition experiments with purified preparations of the membrane-bound hydrogenases of *Thiocapsa roseopersicina* (Gogotov *et al.*, 1978) and *Alcaligenes eutrophus* H16 (Schink & Probst, 1980) and the use of diverse hydrogenases in the chloroplast–hydrogen-evolution system (Rao & Hall, 1979) rather indicated that the so-called 'oxygen-stable' hydrogenases are very O_2-sensitive during the catalytic reaction. On the other hand, the soluble hydrogenase (hydrogen–NAD^+ oxidoreductase, EC 1.12.1.2) of *A. eutrophus* H16, which is also known to be stable in air (Schneider & Schlegel, 1976), during catalytic reaction is not, or only to a negligible extent, inhibited by O_2 (Schneider & Schlegel, 1977; Schneider *et al.*, 1979). In view of the practical importance of O_2-insensitive hydrogenases, the present paper gives more detailed data on stability and reactivity of the soluble *Alcaligenes* hydrogenase in the presence of O_2, and evidence is provided that this enzyme is able to produce superoxide radicals in the presence of O_2 and electron donors.

Materials and methods

Chemicals

The chemicals used were: from Boehringer, glucose oxidase, NADH and cytochrome *c*; from Sigma, superoxide dismutase. All other chemicals were obtained from Merck.

Enzyme preparation

Crude extracts and purified soluble hydrogenase from autotrophically grown cells of *Alcaligenes eutrophus* H16 were prepared as described previously (Schneider & Schlegel, 1976).

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Assay of hydrogenase

Hydrogenase activity was measured spectrophotometrically or manometrically by monitoring the reduction of NAD^+ or the evolution of hydrogen from NADH respectively (Schneider & Schlegel, 1976).

Assay for the detection of superoxide radicals

Hydrogenase at a concentration of 0.1–0.4 mg of protein/ml buffer was provided with hydroxylamine hydrochloride to give a final concentration of 0.5 mM. In the presence of superoxide radicals, hydroxylamine was oxidized to nitrite (Elstner & Heupel, 1976). The formation of nitrite was determined by adding 0.25 ml each of 7 mM-α-naphthylamine and 19 mM-sulphanilic acid solutions [for the preparation of these reagents, see Elstner & Heupel (1976)] to 0.25 ml of the reaction mixture. The components were mixed and after being left at room temperature for 20 min A_{530} was measured.

Results

Stabilization of hydrogenase by oxidation

The observation that the purified soluble hydrogenase from *A. eutrophus* H16 (*Hydrogenomonas* H16) is more stable under air than under H_2 and/or in the presence of NADH (Schneider & Schlegel, 1976) led to the assumption that the instability of the enzyme in crude extracts is due to the presence of potential electron donors. To confirm this hypothesis, the influence of reducing as well as oxidizing reagents on the stability of hydrogenase in crude extracts was examined (Table 1).

Untreated cell extracts kept under air lost about 60% activity within 4 days when stored at 4°C. When kept under H_2 the loss of activity amounted to more than 80%, and the addition of NADH (5 μM) or mercaptoethanol (1 mM) resulted in the complete loss of activity. Strikingly, the enzyme could be stabilized by merely gassing the extracts with O_2. Storage under an atmosphere containing 60% (v/v) O_2 dramatically decreased the activity loss to 15%. Most effective stabilization of hydrogenase was achieved by oxidation with ferricyanide under air. The optimal concentration of ferricyanide depended on the redox conditions and the concentration of reduced nicotinamide nucleotides in the extracts and, therefore, on the conditions under which the

Table 1. *Stability of hydrogenase in crude cell extracts prepared from autotrophically grown cells of Alcaligenes eutrophus H16*

Conditions of storage (4°C)		Loss of activity after 4 days
Atmosphere	Additions	(%)
Air	None	62
H_2	None	84
H_2	NADH (5 μM)	100
N_2	Mercaptoethanol (1 mM)	97
30% O_2/70% N_2	None	50
60% O_2/40% N_2	None	15
Air	Ferricyanide (1 mM)	0

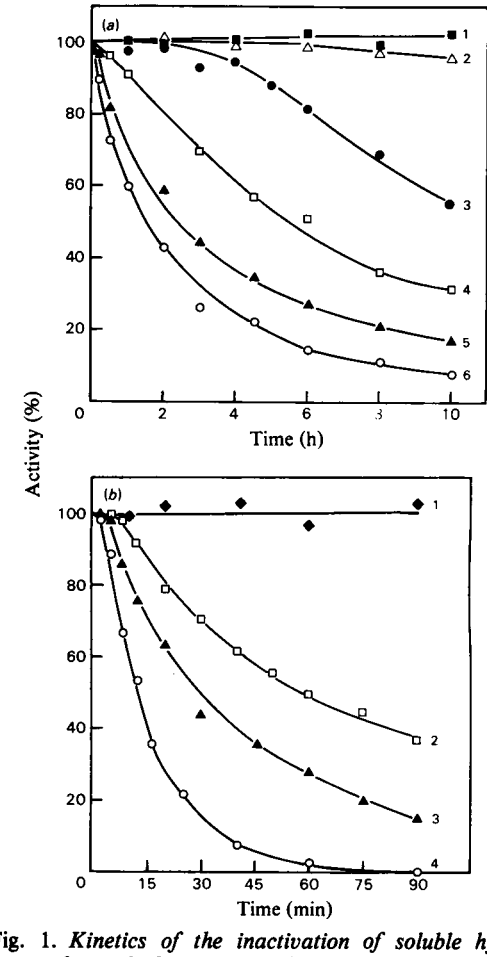

Fig. 1. *Kinetics of the inactivation of soluble hydrogenase from Alcaligenes eutrophus H16 in the presence of NADH, H_2 and various concentrations of O_2*

(*a*) Incubation of hydrogenase (6.3 μg) in 0.15 ml of 50 mM potassium phosphate buffer, pH 7.0 at 4°C: (*b*) incubation of hydrogenase (6.3 μg) in 0.15 ml of 50 mM-Tris/HCl, pH 8.0, at 30°C. The samples that contained 25 μM-NADH were incubated under air (△); 0.1% O_2/99.9% H_2 (●); 5% O_2/95% H_2 (□); 20% O_2/80% H_2 (▲) and 50% O_2/50% H_2 (○). The samples which contained no NADH were incubated under 20% O_2/80% H_2 (■) and air (♦). Samples (10 μl) were taken at time intervals and tested for activity.

cells were cultivated. Whereas extracts of cells grown autotrophically at maximum rates required the addition of only 0.5–1 mM-ferricyanide (as in the experiment presented in Table 1), extracts of cells grown under O_2 limitation required about 10 mM-ferricyanide for enzyme stabilization.

Inactivation kinetics of purified hydrogenase

To explore the causative mechanism of enzyme inactivation, the time course of the inactivation process of the purified hydrogenase was studied (Fig. 1). In the first experiment (Fig. 1*a*, curve 3) conditions were chosen that were routinely applied to treat the hydrogenase under 'reducing conditions'. The enzyme was incubated in a buffer solution (50 mM-potassium phosphate, pH 7.0) containing 25 μM-NADH under H_2 at 4°C. Because of the apparent O_2-stability of the hydrogenase, no precautions were taken to remove O_2 from the H_2 completely. The H_2 used contained about 0.1% (v/v) O_2. Under these conditions the inactivation rate was very low during the first 2–3 h, but was then followed by a phase of acceleration of inactivation. The half-life of the enzyme was 11 h. After 1 day, only a few per cent of residual activity was left. Surprisingly, the higher the O_2 concentration in the H_2/O_2 mixture under which the hydrogenase was stored, the higher was the rate of inactivation. The half-lives in 5% O_2/95% H_2, 20% O_2/80% H_2 and 50% O_2/50% H_2 mixtures were 5, 2.5 and 1.5 h respectively.

In a second set of experiments the enzyme was kept in a buffer solution in which it shows maximum activity (50 mM-Tris/HCl, pH 8.0), and at 30°C instead of incubation under storage conditions (pH 7.0, 4°C). After a short phase of nearly constant activity (in the presence of 5% O_2, 5–8 min; 20% O_2, 2–4 min; and 50% O_2, 1–2 min), the enzyme activity declined rapidly. The inactivation was again dependent on the concentration of O_2 and was about five times faster than the inactivation observed at 4°C and at neutral pH (Fig. 1*b*). The half-lives were, in the presence of 5% O_2, 60 min; 20% O_2, 30 min; 50% O_2, 12 min. Under H_2-free air at 30°C the hydrogenase did not lose any activity within the period (90 min) of the experiment (Fig. 1*b*, curve 1).

These results mean that, in contrast with the function of O_2 as a stabilizer of hydrogenase in extracts from which H_2 was absent or removed, O_2 has, if present concomitantly with H_2 and NADH, a remarkably destabilizing effect. Controls, H_2 and H_2/O_2 (Knallgas) mixtures in the absence of NADH, or catalytic amounts of NADH in the absence of H_2, had no or an insignificant effect on the purified hydrogenase (compare curves 1 and 2 of Fig. 1*a* and Table 2), indicating that each of the compounds used, H_2, NADH and O_2, is required

Table 2. *Production of superoxide radicals by purified hydrogenase and correlated enzyme inactivation under various reducing conditions*

The samples contained 50 mM-potassium phosphate, pH 7.0, 0.5 mM-hydroxylamine hydrochloride and 0.1 mg of hydrogenase in a final volume of 0.25 ml. Further additions and the gas mixtures under which the samples were stored at 4°C are indicated in the Table. After 12 h the hydrogenase solutions were mixed with equal volumes each of α-naphthylamine and sulphanilic acid solutions, and the A_{530} was measured. Parallel samples without hydroxylamine hydrochloride were run for activity determination (NAD^+ reduction).

Conditions of storage (4°C)		A_{530} after 12 h	Hydrogenase activity (ΔA_{365}/min) after 12 h
Atmosphere	Additions		
Air	None	0	0.177
H_2*	None	0	0.178
80% H_2/20% O_2	None	0	0.174
H_2*	25 μM-NADH	0.033	0.143
99.9% H_2/0.1% O_2	25 μM-NADH	0.137	0.090
99% H_2/1% O_2	25 μM-NADH	0.502	0.031
95% H_2/5% O_2	25 μM-NADH	1.420	0.014
80% H_2/20% O_2	25 μM-NADH	1.610	0.001
Air	25 μM-NADH	0.022	0.159
Air	1 mM-NADH	0.415	0.010
99% N_2/1% O_2	1 mM-Dithionite (without hydrogenase)	0.078	—
Air	210 units of glucose oxidase + 10 mM-glucose (without hydrogenase)	0.042	—

* The samples incubated under H_2 were freed from traces of O_2 by flushing the enzyme solution for 1 h with H_2, which was pre-purified by passage through a copper column. The O_2-free NADH solution was added after the gassing procedure.

for complete enzyme inactivation. Singly applied, none of them seems to have the ability to inactivate hydrogenase. These observations suggested that hydrogenase, in the presence of electron donors reacts with molecular O_2 to form superoxide anions ($O_2^{-\cdot}$), which, by their radical character, cause the inactivation of the enzyme.

Evidence for the generation of superoxide radicals by hydrogenase

By the use of hydroxylamine as trapping agent and detector of $O_2^{-\cdot}$ radicals (Elstner & Heupel, 1976), it was demonstrated that hydrogenase is indeed able to produce superoxide radicals. Determination of the nitrite formed from hydroxylamine by oxidation with superoxide radicals turned out to be the most suitable and specific method to detect the radicals in enzyme solutions because this reagent, unlike other $O_2^{-\cdot}$-trapping agents, neither influences enzyme activity nor reacts with hydrogenase itself. If the amounts of nitrite are considered to represent the amounts of superoxide radicals formed, one can draw the conclusion, from the data of Table 2 and also from the comparison of the enzyme-inactivation kinetics (Fig. 1) with the kinetics of superoxide-radical production (Table 3), that fairly strong correlations exist between: (1) the O_2 concentration used in the assays and the amount of generated $O_2^{-\cdot}$ radicals, and (2) the amount of radicals and the degree of enzyme inactivation. The fact that superoxide radicals were even detected in samples that were flushed with O_2-free H_2 for 1 h before addition of NADH (Table 2) is assumed to be due to catalytic amounts of O_2 that are bound to the enzyme and cannot be removed by physical means alone (see the Discussion section). Under air, H_2 or H_2/O_2 mixtures (Knallgas) in the absence of NADH, no superoxide radicals were produced; under air in the presence of 25 μM-NADH, however, small, but easily detectable, amounts were formed, corresponding to the moderate instability of purified hydrogenase under such conditions. Consequently, significant inactivation and increase in superoxide-radical production was observed after the use of a relatively high NADH concentration (1 mM). This means that, apart from H_2, NADH can also be used by the enzyme as electron donor for the reduction of O_2.

To measure the time-dependence of superoxide-radical production and the direct influence of superoxide dismutase on nitrite formation, the hydrogenase was, as under reaction conditions, incubated at 30°C in Tris buffer (50 mM, pH 8.0) containing catalytic amounts of NADH and was saturated with different O_2/H_2 mixtures. After a few minutes, radicals could be detected in each assay, and the rate of their production was again dependent on the O_2 concentration (Table 3). In the presence of

Table 3. *Time-dependent production of superoxide radicals at 30°C*

The reaction mixture (1 ml) saturated with H_2/O_2 (Knallgas) mixtures containing O_2 concentrations as indicated in the Table, contained 50 mM-Tris/HCl, pH 8.0, 0.1 mg of purified hydrogenase, 25 μM-NADH, 0.5 mM-hydroxylamine hydrochloride ± 2 mg (5800 units) of superoxide dismutase and was incubated in glass tubes at 30°C. The nitrite concentration of samples taken at different times were determined as described in the Materials and methods section.

Incubation time	5% O_2		5% O_2 + superoxide dismutase		20% O_2		50% O_2		50% O_2 + superoxide dismutase	
	A_{530}	[Nitrite] (μM)	A_{530}	[Nitrite] (μM)	A_{530}	[Nitrite] (μM)	A_{530}	[Nitrite] (μM)	A_{530}	[Nitrite] (μM)
20 min	0.043	2	0.012	0.6	0.325	17	1.271	78	0.090	5
1 h	0.108	6.5	0.016	0.7	0.471	25	1.633	150	0.133	7
2 h	0.239	13	0.023	1	0.797	43	1.750	200	0.147	8
4 h	0.039	18	0.034	1.7	1.035	59	1.750	200	0.352	19

5% O_2, nitrite was formed at a rate of 2 μM/20 min over a period of about 2 h. The initial rate of nitrite formation in the presence of 20% and 50% O_2 was markedly higher (17 and 78 μM/20 min respectively), but decreased afterwards, obviously due to enzyme decay. If superoxide dismutase was present in the reaction mixtures the formation of nitrite was inhibited by 90–95%. The relief exerted by superoxide dismutase is considered to be confirmatory evidence for superoxide radicals being generated by hydrogenase and responsible for the oxidation of hydroxylamine.

Stabilization of hydrogenase under reducing conditions

The stabilization experiments were performed with an enzyme preparation that lost more than 90% activity after 1 day under standard reducing conditions, i.e. in the presence of NADH under H_2 containing 0.1% O_2 (Table 4).

As hydrogenase might also produce, in addition to superoxide anions, H_2O_2, which can itself interact with $O_2^{-\cdot}$ to generate the highly reactive hydroxyl radicals ($OH^\cdot$) and singlet oxygen molecules ($^1O_2$) (Kellog & Fridovich, 1977), we first examined whether the inactivation of hydrogenase is partly caused by these or one of these oxygen species. H_2O_2, up to a concentration of 1 mM, did not affect hydrogenase at all. This is consistent with the observation that the enzyme was not stabilized by catalase. Benzoate and formate, used as $OH^\cdot$ scavengers, and histidine, which is known to react rapidly with singlet oxygen, had no protective effect on hydrogenase either. These results permitted us to conclude that hydrogenase is indeed inactivated merely by self-produced superoxide radicals. In this case the addition of reagents and enzymes removing O_2 or having $O_2^{-\cdot}$-trapping activity should result in enzyme stabilization. The results of respective experiments shown in Table 4 demonstrate that only about half of the original activity was retained when the enzyme was incubated in the presence of dithionite or glucose and glucose oxidase for 24 h. This becomes understandable if we realize that, as shown in control assays (compare Table 2), dithionite as well as glucose oxidase [which is known to contain two molecules of FAD/enzyme molecule (Kusai *et al.*, 1970)] are able to reduce oxygen by the univalent pathway, thus producing superoxide radicals directly and independently of the radical formation by hydrogenase. In addition, in the case of glucose oxidase, it might be that the affinity of this enzyme for O_2 is too low to prevent the reaction of hydrogenase with molecular O_2 completely. A better, but not perfect, protection against enzyme inactivation was achieved by adding compounds that react with superoxide radicals directly. After 1 day in the presence of cytochrome *c*, the activity was 67%; in the presence of superoxide dismutase, which proved to be the best stabilizer, it was 72%. Glucose oxidase in the absence of glucose and bovine serum albumin, even if present in the assay mixture at very high concentrations (10 mg/0.25 ml) did not influence hydrogenase stability significantly, demonstrating conclusively that the stabilizing effect of cytochrome *c* and superoxide dismutase is really due to their scavenging the superoxide radicals. The finding that hydrogenase was not fully stabilized in the presence of superoxide dismutase may be due to the competition of both enzymes, hydrogenase and dismutase, for the superoxide radicals, the minor proportion of which react with hydrogenase more rapidly than with superoxide dismutase. The combination of a more extensive pretreatment of the enzyme with O_2-free H_2 to remove dissolved free O_2 and of the addition of superoxide dismutase to the enzyme solution (see Table 4) finally proved to provide the most favourable conditions for stabilizing hydrogenase in the presence of H_2 and NADH.

Table 4. *Stabilization of hydrogenase under reducing conditions*

Assay conditions were as described for Table 2. The samples incubated under 99.9% H_2/0.1% O_2 were provided with NADH (25 μM) before the gassing procedure (15 min); the samples incubated under O_2-free H_2 (pre-purified by a copper column) were provided with NADH after gassing (1 h).

Conditions of storage (4°C)		Hydrogenase activity after 24 h (%)
Atmosphere	Additions	
Air	None	100
	H_2O_2 (1 mM)	100
	H_2O_2 (10 mM)	85
99.9% H_2/ 0.1% O_2	None	7
	Formate (1 mM)	10
	Benzoate (1 mM)	9
	Histidine (1 mM)	7
	Catalase (180 units)	6
	Bovine serum albumin (1 mg)	9
	Bovine serum albumin (10 mg)	18
	Glucose oxidase (105 units)	8
	Glucose oxidase (105 units) + glucose (5 mM)	53
	Dithionite (1 mM)	62
	Hydroxylamine (2 mM)	48
	Cytochrome *c* (1 mg)	67
	Superoxide dismutase (2900 units)	72
100% H_2	None	81
	Cytochrome *c* (1 mg)	95
	Superoxide dismutase (2900 units)	97

O_2-tolerance of hydrogenase during reaction

To test the direct influence of O_2 and superoxide radicals on hydrogenase during the catalytic reac-

tion, the enzyme activity was measured in the presence of different O_2 concentrations. Neither the activity of H_2 uptake (NAD^+ reduction) nor that of H_2 evolution (from NADH) was significantly inhibited by O_2 (Table 5). After equilibration of the reaction mixture with Knallgas containing up to 10% (v/v) O_2, almost no inhibition was detectable at all, and the increase of the proportion of O_2 in the gas mixture to 60% (equivalent to 0.7 mM-O_2) in the assay system diminished the activity by only about 20%. There was no difference in the maximum reaction rate and the inhibition effect whether the oxidized (oxygenated) enzyme (see Schneider & Schlegel, 1976) or the enzyme that was reduced before addition was used. With the latter enzyme, all reactions started without a lag period. With the oxidized enzyme, as it had been obtained by purifying it under aerobic conditions, there was no lag period in the H_2-evolution test; however, a lag was observed in the reduction of NAD^+. This lag phase lasted about 30 s in the O_2-free assay, 3.5 min in the presence of 5% O_2 and 6 min in the presence of 60% O_2. Apparently, molecular O_2 merely retards, but does not prevent and inhibit, the reaction. The superoxide radicals that are formed do not inactivate hydrogenase immediately. The inactivation process caused by the action of superoxide radicals is a relatively slow process and starts only after a few minutes incubation, even at 30°C (see Fig. 1*b*), and it therefore does not affect hydrogenase during the short period of enzyme assay.

Table 5. *Hydrogenase activity in the presence of O_2*
Before the reaction was started by addition of enzyme, each reaction mixture was flushed for 20 min with gas mixtures containing a constant proportion of H_2 (4 parts by volume) and various proportions of O_2 (0–6 parts by volume) with N_2 as expander. Further conditions for the enzyme assays were as described by Schneider & Schlegel (1976). Abbreviation used: n.d., not determined.

$[O_2]$ (%, v/v) in the gas mixture	$[O_2]$ in the reaction mixture (mM)	Hydrogenase activity* (%)	
		NAD^+-reduction	H_2 production from NADH
0	0	100	100
1	0.012	100	100
5	0.060	100	n.d.
10	0.120	95	n.d.
20	0.230	92	87
40	0.465	87	n.d.
60	0.700	82	80

* The values of relative hydrogenase activity are referred to maximum rates during reaction. Maximum reaction rates were reached either immediately after start of reaction (H_2 production) or after a lag phase (NAD^+ reduction) and remained constant over a period of 2–3 min. For details of reaction kinetics, see Schneider & Schlegel (1976).

Discussion

In the past, hydrogenase has repeatedly been characterized as a very O_2-sensitive enzyme that is reversibly oxygenated by O_2 as well as irreversibly inactivated by oxidation with oxygen (Mortenson & Chen, 1974). The bidirectional hydrogenase of *Clostridium pasteurianum*, often considered as the 'classical' model and reference enzyme, is completely inactivated after exposure to air for 60 min (Nakos & Mortenson, 1971). During the last few years, however, it has been established that this extreme O_2-sensitivity is not typical but rather shared by only a minority of hydrogenases, i.e. the enzymes of *Megasphaera elsdenii* (Mayhew *et al.*, 1978), *Escherichia coli* (Adams & Hall, 1979), *Rhizobium japonicum* (Arp & Burris, 1979) and green algae (Kessler, 1978; Erbes *et al.*, 1979). In contrast, many other hydrogenases isolated and purified from *Chromatium* (Gitlitz & Krasna, 1975), *Rhodospirillum rubrum* (Adams & Hall, 1977), *Thiocapsa roseopersicina* (Gogotov *et al.*, 1978), various *Desulfovibrio* species (Van der Westen *et al.*, 1978; Hatchikian *et al.*, 1978), *Proteus mirabilis* (Schoenmaker *et al.*, 1979), *Alcaligenes eutrophus* (Schneider & Schlegel, 1976; Schink & Schlegel, 1979), several other hydrogen bacteria (Bone, 1963; Aggag & Schlegel, 1974; Weiss *et al.*, 1980) and *Anabaena cylindrica* (Hallenbeck & Benemann, 1978) have been described as being O_2-stable or relatively O_2-insensitive. It should, however, be noted that the term 'O_2-insensitivity' was ill-defined, and in most recent papers referred only to enzyme stability under conditions of storage, and not to the possible influence of O_2 on the functioning enzyme. In spite of its practical importance, the problem of whether hydrogenase is active at all in the presence of O_2 and whether the activity is decreased or reversibly inhibited under reaction conditions was seldom investigated. There are, however, a few reports indicating that the activity of many 'O_2-stable' hydrogenases is either strongly inhibited by O_2 or that the enzymes are kept in a completely inactive form if O_2 is not removed from the assay. Fisher *et al.* (1954), with whole cells and cell-free extracts of *Proteus vulgaris*, were the first to demonstrate the inhibition of hydrogenase by oxygenation. They postulated the formation of an inactive hydrogenase–O_2–complex that is in equilibrium with the active O_2-free enzyme. Gogotov *et al.* (1978), studying the purified hydrogenase of *Thiocapsa roseopersicina*, reported that, if during the enzyme reaction 1% O_2 was introduced into the gas phase, the enzyme activity was immediately and

completely inhibited. Finally, Schink & Probst (1980) found that the purified membrane-bound hydrogenase of *A. eutrophus*, which proved to be very stable when stored under air (Schink & Schlegel, 1979), is also markedly inhibited by oxygen [50% inhibition in the presence of 35 μM (~3%) O_2]. The inhibition was shown to be competitive with respect to H_2, indicating that, in accordance with the postulation of Fisher *et al.* (1954), O_2 combines reversibly with the active centre of hydrogenase.

The soluble hydrogenase of *A. eutrophus* H16 was characterized as a complex iron–sulphur enzyme that holds a rather exceptional position among the hydrogenases so far described. It is able to reduce NAD^+ directly, exhibits diaphorase activity, and contains bound FMN (Schneider & Schlegel, 1978) as well as two types of iron–sulphur centres, the 2Fe–2S type and the 4Fe–4S type (Schneider *et al.*, 1979). The use of O_2 as an enzyme stabilizer in extract preparations, the extraordinary O_2-tolerance under reaction conditions, and the ability to react with O_2 as electron acceptor and to produce superoxide radicals are further unique properties of this hydrogenase. From these data and from the kinetics of NAD^+ reduction and the elimination of the lag period by enzyme reduction or by removal of O_2 (compare Schneider & Schlegel, 1976), one can conclude that the soluble *Alcaligenes* hydrogenase, in spite of the stated 'oxygen tolerance', does interact with O_2 and probably in different ways and at different sites.

The physiologically oxidized hydrogenase binds O_2 to give a non-reactive but very stable enzyme form. On the one hand, this enzyme-O_2 complex appears to be more stable than that of other hydrogenases. As shown by stability measurements and by light absorption and e.p.r. spectra (Schneider *et al.*, 1979), hydrogenase does not become reduced even after extensive treatment with H_2, and it is unable, apparently because the H_2-binding site remains blocked by O_2, to react with H_2 and to reduce O_2 with electrons derived from H_2. On the other hand, if catalytic amounts of a reduced electron carrier, such as NADH, are present, hydrogenase is transformed into the active state and reduced by H_2 very rapidly. This activation occurs at a lower rate, but almost to completion, even in the presence of high O_2 concentrations. The enzyme, once activated, remains active and is not, or only very slightly, inhibited by O_2 during enzymic catalysis. This characteristic behaviour of the enzyme was confirmed by using the enzyme in the chloroplast–H_2-evolution system. So far, the soluble hydrogenase of *A. eutrophus* H16 is the only hydrogenase that evolves H_2 from water (with NADH or Methyl Viologen as electron carrier) at the same rate regardless of whether O_2 scavengers are present or not (Rao & Hall, 1979; Rao *et al.*, 1979). The ability to evolve H_2 in the presence of O_2 has recently been demonstrated also for the Russian strain Z1 of *A. eutrophus* (Zorin *et al.*, 1979).

One of the unsolved questions concerns the mechanism by which oxygenation is reversed and the hydrogenase becomes re-activated. As the oxygenated enzyme is not activated by H_2 and the reduced, active enzyme is not inhibited by O_2, a typical competitive interaction between O_2 and H_2, such as postulated for the membrane-bound hydrogenase of *A. eutrophus*, is highly improbable. It seems rather likely that O_2 molecules, instead of being removed by competing H_2 molecules, are reduced by electrons derived from NADH and then are dissociated from the enzyme. Alternatively, it cannot be excluded that the essential role of NADH in the re-activation mechanism is to cause a conformational change of the enzyme leading to dissociation of O_2. The lack of strong inhibitory effects of O_2 on hydrogenase activity suggests that O_2 has a strong affinity for the H_2-binding centre of the oxidized hydrogenase but no, or at best low, affinity, for the H_2-binding centre of the activated and reduced enzyme form.

As H_2 can be used by hydrogenase as electron donor for O_2 reduction to produce superoxide radicals, there must exist, apart from the site that is oxygenated before enzyme reduction, at least one other site that reacts with O_2 after reduction. This assumption is supported by the earlier finding (Schneider & Schlegel, 1976) that hydrogenase is able to use, in addition to NAD^+ and many other redox compounds, molecular O_2 as electron acceptor. The Knallgas (95% H_2/5% O_2)-uptake rate was determined to be 2% of the H_2-uptake rate with NAD^+ as acceptor. This low rate of O_2 reduction corresponds to the low inhibitory effect exerted by O_2. It is therefore assumed that the inhibitory effect of O_2 results from O_2 competing with NAD^+ (or with protons in the H_2-evolution assay) for electrons and not from O_2 competing with H_2 for the H_2-binding site.

The question that remains is: 'which site of the reduced hydrogenase does react with O_2?' The generation of superoxide radical anions has been described for several iron–sulphur proteins, including ferredoxins (Misra & Fridovich, 1971) and flavin-containing enzyme systems, such as xanthine oxidase (McCord & Fridovich, 1969). Both non-haem iron and flavin groups have been postulated as sites of O_2 reduction to $O_2^{-\cdot}$ (Misra & Fridovich, 1972). Whereas ferredoxins with two 4Fe–4S centres were shown to produce only superoxide anions (Orme-Johnson & Beinert, 1969), the oxidation of 2Fe–2S plant ferredoxin produces mainly peroxide (Allen, 1975). Flavins are able to transfer electrons singly or in pairs and therefore to perform

univalent reduction of O_2 to the superoxide radical as well as bivalent reduction to peroxide. Generally, however, iron–sulphur centres appear to be more effective O_2 reductants than do flavins (Misra & Fridovich, 1972). As soluble hydrogenase of *A. eutrophus* contains FMN, 2Fe–2S centres and 4Fe–4S centres, one can expect that not only one specific reaction site but that all three electron-transferring components are involved in $O_2^{-\cdot}$ production and that, in addition, H_2O_2 is formed.

The present paper is the first report of the production of superoxide radicals by a bacterial hydrogenase, although all hydrogenases so far studied contain iron–sulphur centres (see Schlegel & Schneider, 1978), and some of them, the membrane-bound hydrogenases of *Chromatium* (Kakuno *et al.*, 1977), *A. eutrophus* (Schink & Schlegel, 1979) and *Methanobacterium* strain G2R (McKellar & Sprott, 1979), were found to be unstable under reducing conditions or inhibited by reductants respectively.

It was suggested from earlier work on soluble *Alcaligenes* hydrogenase (Schneider & Schlegel, 1976) that the instability of the enzyme under reducing conditions was due to splitting of (a) disulphide bond(s) by reduction leading to unfolding of the protein. However, from the present study it has been established conclusively that enzyme inactivation is not caused by reduction but by the action of self-produced superoxide radicals. $O_2^{-\cdot}$ radicals are reported to be able to cause extensive damage to various biological structures such as proteins (Michelson, 1977), but little is known about the sites of action of superoxide radicals and the chemical effects by which protein molecules are modified. One possible reaction with a protein is the oxidation of SH groups by superoxide anions (Fridovich, 1976). Whether $O_2^{-\cdot}$ also interacts directly with iron–sulphur centres has not yet been elucidated. However, as it has been observed that the process of hydrogenase inactivation is accompanied by the loss of e.p.r. signals as well as of absorption in the iron–sulphur chromophore region (K. Schneider & R. Cammack, unpublished work), it seems possible that the iron–sulphur centres of hydrogenase are destroyed by the action of superoxide radicals.

The hydrogenase of *Desulfovibrio vulgaris* was reported to be very stable under air in the oxidized form as isolated, but to be inactivated by air after reduction of the enzyme (Mayhew *et al.*, 1978; Van der Westen *et al.*, 1980). This kind of enzyme inactivation seems to resemble that observed for the reduced soluble *Alcaligenes* hydrogenase in the presence of O_2. There are, however, significant differences in the behaviour of the enzymes pointing to different mechanisms of inactivation. (1) In contrast with the *Desulfovibrio* enzyme, removal of H_2 and exposure to air tends to stabilize rather than inactivate the hydrogenase of *A. eutrophus*. (2) For inactivation of the latter enzyme, the simultaneous presence of O_2 and reductants, not a preceding reduction followed by reoxidation, is required. (3) Whereas the *Alcaligenes* hydrogenase is inactivated comparatively slowly and can be protected by superoxide dismutase, the inactivation of the *Desulfovibrio* hydrogenase is a much faster process (57% inactivation after 2 min at room temperature) and cannot be prevented by superoxide dismutase. The inactivation of the *Desulfovibrio* hydrogenase, therefore, is apparently caused not by superoxide radicals but by conversion into an extremely O_2-sensitive form by reduction.

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