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CHELATING AGENTS PROTECT HYDROGENASE AGAINST OXYGEN INACTIVATION

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Summary

The effect of chelation on rate of air inactivation of hydrogenase from *Clostridium pasteurianum* has been investigated. All chelating agents used, whether water-soluble or water-insoluble, afforded protection against oxygen inactivation. EDTA appeared to be the most effective. Thus, in the absence of EDTA, hydrogenase in aqueous solution was nearly totally inactivated after 1 hour incubation in air, whereas 0.5 M EDTA (which did not affect significantly catalytic activity) allowed 41% retention of the initial activity even after 3 days incubation.

Like many other iron-sulfur proteins [1] hydrogenase is oxygen-labile [2]. Because of the potential practical significance of hydrogenase for construction of solar energy conversion systems [3—6], protection of the enzyme against inactivation by air oxygen may be an important problem in applied bioenergetics.

In a previous communication [7], we developed a principle for stabilization of oxygen-labile enzymes, which exploited the "salting out" of oxygen from the microenvironment of enzymes immobilized on polyionic supports. We suggest herein another and independent approach.

Based on the investigation of the mechanism of oxygen inactivation of ferredoxins, it had been suggested [8] that O_2 sensitivity of many non-heme iron-sulfur proteins was related to the oxidation of their mercaptide-sulfide constituents. Few, if any, equally plausible alternatives seem to exist. From studies on oxidation of sulphydryl compounds by molecular oxygen (see Refs. 9—13 for reviews) it is known that this process accelerates markedly in the presence of even trace amounts of such metal ions as iron, copper, manganese, etc. which interact with thiol groups to form relatively easily oxidizable complexes. Chelating agents, therefore, may prevent oxidation. Hence, attempts to use chelating reagents for protection of iron-sulfur enzymes against oxygen

inactivation are indicated. We report results which demonstrate that chelating compounds, both water-soluble (EDTA, sulfosalicyclic acid) and water-insoluble (chelating resin Chelex 100) enhance greatly oxygen stability of the O_2 labile enzyme, hydrogenase from *Clostridium pasteurianum*.

Commercially available compounds were obtained as follows: from Mann Research Laboratories, methyl viologen and α, α'-dipyridyl; from Fisher Scientific Co., sodium dithionite (reagent grade); from Mallinckrodt Chemical Works, EDTA, mannitol, sodium citrate (all reagent grade); from Sigma Chemical Co., 5-sulfosalicyclic acid, *o*-phenanthroline, catalase (EC 1.11.1.6) from beef liver (2 x cryst.), glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, superoxide dismutase (EC 1.15.1.1) from bovine blood, β-D(+)glucose, bovine albumin (cryst. and lyophil.) and β-mercaptoethanol; and from Bio-Rad Laboratories, chelating resin Chelex 100 (analyt. grade).

Partial purification of hydrogenase (hydrogen: ferredoxin oxido reductase, EC 1.12.7.1) from *C. pasteurianum* followed the method of Nakos and Mortenson [14] with some modifications as previously described [7]. The specific activity of the final preparation was essentially that of the enzyme preparation described previously [7,15].

For routine hydrogenase assay, we used enzyme-catalyzed H_2 evolution, mediated by methyl viologen, maintained reduced by sodium dithionite. The rate of gas evolution was determined with a Clark-type electrode [16] calibrated against H_2-saturated aqueous solutions. In a typical experiment at room temperature (approx. 22° C), 1.5 ml of 50 mM Tris-HCl buffer solution (pH 8), containing 1 mM methyl viologen and 15 mM sodium dithionite, was incubated with 0.05—0.2 ml of enzyme solution.

The time course of air inactivation was measured as follows: 5 ml of 0.01 M phosphate buffer (pH 8) containing 0.1 ml of hydrogenase solution (10 mg of protein/ml) was stirred (room temperature) in an open beaker and aliquots were withdrawn for enzyme assay. For enzyme solutions containing Chelex resin (1 g), aliquots of the supernatant were withdrawn and assayed for hydrogenase activity.

Air lability of clostridial hydrogenase is shown in Fig. 1, curve a. This inactivation requires access of air oxygen because under an atmosphere of argon or hydrogen, or under air but in the presence of sodium dithionite (oxygen scavenger), hydrogenase is stable for many hours.

Oxygen inactivation of hydrogenase from *C. pasteurianum* is essentially irreversible—significant reactivation of the enzyme cannot be achieved, either by replacement of oxygen by argon or hydrogen, or by subsequent incubation of hydrogenase with mercaptoethanol or sodium dithionite. This fact has been shown from previous studies [2,17].

Oxidation of many organic compounds in aqueous solutions occurs through free radical mechanisms which may involve participation of OH radicals. To check this possibility for oxygen inactivation of hydrogenase, we have studied the effect of mannitol (0.1 M) and formate (0.1 M) (known scavengers of OH radicals [18]) on the process rate. As seen from Fig. 1, curves b and c, these reagents affected only slightly the rate of inactivation. Another possibility (inactivation of hydrogenase under the action of superoxide radicals, which could form during the oxidation of many compounds

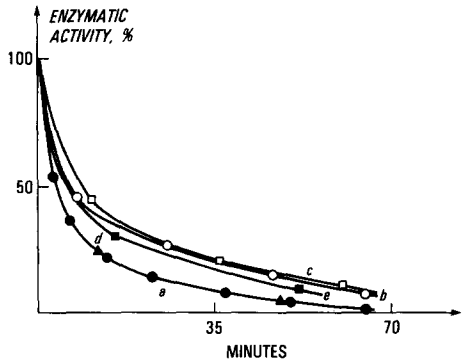

Fig. 1. The effect of different compounds on the rate of air inactivation of clostridial hydrogenase. a, buffer (●); b, 0.5 M mannitol (○); c, 0.5 M formate (□); d, 110 units/ml superoxide dismutase (▲); e, 20 000 units/ml catalase (■). Conditions: 0.01 M phosphate (pH 8.0), stirring under air; for other conditions see Materials and Methods.

[28], particularly some impurities in the enzyme preparation) appeared excluded because (Fig. 1, curve d) addition of superoxide dismutase, catalyzing decomposition of O_2^-, had essentially no effect on the inactivation rate. The participation of hydrogen peroxide also seemed unlikely because catalase did not decelerate the process (Fig. 1, curve e). However, the system glucose oxidase + glucose + catalase (11 units/ml, 30 mM and 20 000 units/ml, respectively, at pH 6.0) (an effective scavenger of molecular oxygen) sharply diminished the inactivation rate. These results indicate it likely that molecular oxygen is directly involved in the rate-limiting stage of the enzyme inactivation.

By analogy with other iron-sulfur proteins [8] we may suggest that oxidation of the mercaptide-sulfide moiety of hydrogenase is responsible for oxygen inactivation of the enzyme. Hence, chelating agents should decelerate air inactivation.

The effect of different chelating reagents [19] (EDTA (0.5 M), sulfosalicylic acid (0.5 M), *o*-phenanthroline (0.01 M), α, α'-dipyridyl (0.01 M), sodium citrate (0.5 M) and water-insoluble chelating resin Chelex 100 (0.2 g/ml) on the oxygen inactivation of clostridial hydrogenase is shown in Figs. 2

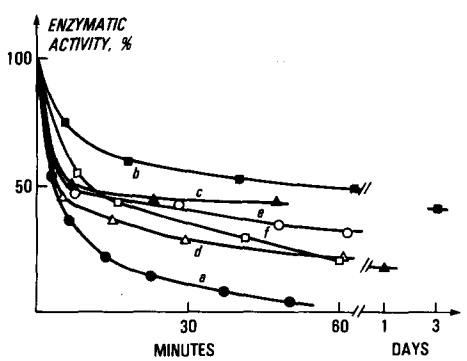

Fig. 2. The effect of different chelating agents on the rate of air inactivation of clostridial hydrogenase. a, buffer (●); b, 0.5 M EDTA (■); c, 0.5 M sulfosalicyclic acid (▲); d, 0.01 M *o*-phenanthroline in 5% ethanol (△); e, 0.01 M α,α'-dipyridyl in 5% ethanol (○); f, 0.5 M sodium citrate (□). Conditions: 0.01 M phosphate (pH 8), stirring under air; for other conditions see Materials and Methods.

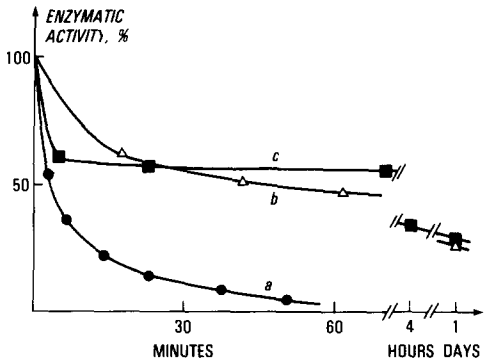

Fig. 3. The effect of bovine albumin (50 mg/ml) and chelating resin Chelex 100 (0.2 g/ml) on the rate of air inactivation of clostridial hydrogenase. a, buffer (●); b, chelex (△); c, albumin (■). Conditions: 0.01 M phosphate (pH 8.0), stirring under air; for other conditions see Materials and Methods.

(curves b—f) and 3 (curve b). One can see that almost all these chelating agents stabilize the enzyme against the inactivation, but to different extents. In addition, bovine albumin (10—50 mg/ml) protects the enzyme against oxygen inactivation (Fig. 3, curve c), also owing perhaps to its chelating properties. The concentrations used essentially exert no effect on hydrogenase activity.

Among all the compounds studied, EDTA exhibits the greatest protective effect (Fig. 2, curve b): whereas in 0.01 M phosphate buffer (pH 8) 1 hour incubation of hydrogenase solution under air leads to nearly complete inactivation of the enzyme (Fig. 2, curve a), the addition of EDTA (final concentration 0.5 M) causes retention of 41% original activity even after 3 days. A progressive decrease in EDTA concentration results in lowered protection (Table I). One may conclude that the concentration of metal ions catalyzing the oxidation process may be quite large in the enzyme incubation mixture. The presence of metal ions firmly bound to protein in preparation of hydrogenase could be considered as an alternative possibility. For example, this metal might be iron from damaged iron-sulfur cluster of inactivated hydrogenase. It has been shown that certain metal complexes are able to catalyze the O_2 oxidation of thiols [10,12]. It may be possible that the protein bound metals could catalyze the oxidation of the mercaptide-sulfide system

TABLE I

THE EFFECT OF EDTA ON THE OXYGEN STABILITY OF CLOSTRIDIAL HYDROGENASE

Conditions: 0.01 M phosphate buffer (pH 8.0), stirring under air; for other conditions see Materials and Methods.

EDTA concentration (mM)	Relative enzymatic activity after certain time of incubation under air, %			
	15 min	1 h	4 h	3 days
0	20	2	0	0
0.5	26	3	0	0
10	40	33	25	6
100	50	42	40	29
500	62	50	44	41

in hydrogenase but they are hardly accessible for the chelation by EDTA and therefore higher concentration of EDTA would be required for the protection of the enzyme against oxygen inactivation.

It is also quite possible that iron atoms of iron-sulfur clusters of active hydrogenase are capable of intramolecular catalysis of O_2 oxidation of the mercaptide-sulfide system of the enzyme, i.e. the same effect of iron of the active center of hydrogenase which activates the mercaptide-sulfide system for the performance of its catalytic function [2], also increases the reactivity of this system towards oxygen. If so, a correlation should exist between the enzymatic activity of different hydrogenases and their O_2 stability. Literature values for specific activities towards reduced methyl viologen and half-times of O_2 inactivation of hydrogenases from *C. pasteurianum* [20, 7], *Desulfovibrio vulgaris* [21], *Desulfovibrio gigas* [22], *Alcaligenes eutrophus* [23], *Thiocapsa roseopersicina* [24], *Rhodospirillum rubrum* [25] and *Chromatium vinosum* [26, 27] were compared and the following qualitative correlation was found: the higher the catalytic activity of the enzyme the lower its oxygen stability. This correlation, if upheld by further experimentation, allows the prediction that hydrogenases possessing both very high enzymatic activity and O_2 insensitivity cannot exist.

In one previous study [7] we demonstrated that adsorption of clostridial hydrogenase on polyethyleneimine attached to cellulose greatly stabilized the enzyme against air (oxygen) inactivation owing to salting out of oxygen) from the immobilized enzyme microenvironment. Because of the eluting action of chelators (EDTA at concentrations greater than 10 mM desorbs hydrogenase) chelation and adsorption are hardly compatible. Nevertheless, even 10 mM EDTA yielded some addidtional stabilization of the immobilized enzyme.

It is worth noting from a phenomenological viewpoint that as was found in [20], EDTA and bovine albumin protect clostridial hydrogenase against inactivation under anaerobic conditions, which was ascribed to poisoning by impurities in a commercial preparation of the substrate, methylene blue.

The stabilization of clostridial hydrogenase by chelating agents described in the present work shows that metal-catalyzed oxidation of sulphydrol groups of the enzyme can provide an explanation for protective action of chelation which may be of practical importance, not only for applications involving use of air-labile hydrogenases, but also for other iron-sulfur oxygen unstable enzymes.

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