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THE EFFECT OF ELECTRON CARRIERS AND OTHER LIGANDS ON OXYGEN STABILITY OF CLOSTRIDIAL HYDROGENASE

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Summary

The effects of various electron carriers, a substrate (H_2) and a reversible inhibitor (CO) on the rate of irreversible oxygen inactivation of clostridial hydrogenase (ferredoxin: H^+ oxidoreductase, EC 1.18.3.1) have been studied kinetically. Some electron carriers (e.g., clostridial ferredoxin and methyl viologen) greatly stabilize the enzyme, some (FAD, FMN) drastically reduce its stability, while others (benzyl viologen and methylene blue) only slightly alter the stability. Competitive experiments indicate that stabilizers and destabilizers do not compete with each other for binding with the active center of hydrogenase. Hydrogen and CO do not affect the rate of the oxygen inactivation. On the basis of the results obtained herein and kinetic data on hydrogenase catalysis from the literature, it is concluded that the active center of this hydrogenase comprises at least three different independent subsites. The first one (presumably an iron atom of the iron-sulfur cluster) binds H_2 and CO and does not contribute to the oxygen stability. The second one binds stabilizers like methyl viologen while the third one binds destabilizers like FMN and FAD.

Introduction

Studies on stability and stabilization of enzymes are of importance for both pure and applied enzymology. Among various methods of enzyme stabilization

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[1—4], stabilization by substrates and other ligands occupies a special place, inasmuch as these compounds are inherent in enzymatic systems. Despite the obvious physiological and biotechnological significance, mechanisms of enzyme stabilization by effectors are not yet well understood [5].

In principle, binding of ligands can lead to stabilization or destabilization or to no effect at all. Numerous examples of these effects in enzyme inactivation brought about by heat, pH, organic solvents, neutral salts and other denaturants are cited in the literature (see Refs. 2, 6 and 7 for a review). However, why some ligands stabilize enzymes while others do not in most instances remains unclear. In the majority of cases, the effect of a single ligand on the stability of an enzyme has been studied. We believe that most interesting information could be obtained by studying the effect of a wide range of effectors. Therefore, in the present work we have investigated the effect of various electron carriers, a substrate and an inhibitor on the oxygen stability of the enzyme hydrogenase (ferredoxin: H^+ oxidoreductase, EC 1.18.3.1).

Hydrogenase is an iron-sulfur protein which reversibly activates molecular hydrogen. It is a key enzyme for proposed 'artificial' solar energy bioconversion [9,10]. Hydrogenase also can be used for the regeneration of coenzymes [8]. However, biotechnological applications of this enzyme are severely limited because of its instability [10,11]. Although some effective methods for stabilization of hydrogenase against oxygen inactivation have been developed [12,13], alternative approaches are highly desirable. Hence, stabilization of hydrogenase by inherent components of the enzymatic process (electron carriers, substrates, inhibitors) would be of definite practical interest.

Experimental

Materials and Methods

Commercially available compounds were obtained as follows: from Sigma Chemical Company, methyl viologen, methylene blue, neutral red, 2,6-dichlorophenol-indophenol, phenazine methosulfate, FMN, FAD, NADH and NAD^+; from British Drug Houses, benzyl viologen and phenosafranine; from Aldrich Chemical Company, thionin, 1,4-dibromobutene and Janus green; from Eastman, 4,4′-bipyridyl; from Fisher Scientific Company, sodium dithionite. Hydrogen, oxygen, argon and carbon monoxide were purchased from Matheson Gas Products. All other chemicals used were of analytical grade.

Polybutenyl viologen was synthesized by reacting equimolar amounts of 4,4′-bipyridyl and 1,4-dibromobutene in acetonitrile by the method of Factor and Heinsohn [14].

Partial purification of hydrogenase from *Clostridium pasteurianum* followed the method of Chen and Mortenson [15] with some modifications as previously described [12]. The purification was carried out under argon in the presence of trace amounts of sodium dithionite and included heat treatment (at 55°C), DEAE-cellulose chromatography and hydroxyapatite chromatography. The recovery of total hydrogenase activity after these stages was 99, 84 and 64%, respectively. That is, no significant inactivation of the enzyme during purification occurred. The specific activity of the hydrogenase preparations obtained by this technique was usually about 5 μmol H_2/min per mg of protein

using the assay described below. Assuming that purified hydrogenase obtained [15] was 100% pure, the purity of our preparation of the enzyme is about 3%. Ferredoxin from *C. pasteurianum* was purified by the method of Mortenson [16]. A millimolar extinction coefficient (at 390 nm with 1 cm light path) of 30 was used in determining the concentration of the protein.

Hydrogenase assay

Hydrogenase was routinely assayed by enzyme-catalyzed evolution of H_2 from sodium dithionite-reduced methyl viologen [12]. The rate of the gas evolution was determined with a membrane-covered polarographic dissolved oxygen electrode (Clark electrode) calibrated against H_2-saturated aqueous solutions. In a typical experiment, 1.5 ml of 50 mM Tris-HCl buffer solution (pH 8.0)/1 mM methyl viologen/15 mM sodium dithionite was equilibrated at 25°C. 0.05—0.2 ml of the enzyme solution (in the range of concentrations from 0.1—5 mg/ml) was added and the kinetics of the reaction were followed instantaneously with a recorder. In the case of the partly purified hydrogenase preparation, no measurable H_2 evolution occurred in the presence of sodium dithionite alone.

Oxygen inactivation of hydrogenase

The kinetics of oxygen inactivation of the enzyme in the presence and in the absence of added ligands were studied as follows: 2—5 ml of 50 mM Tris-HCl buffer solution (pH 8.0)/0.1 ml hydrogenase (0.1—3.0 mg/ml) were stirred in the dark at room temperature in an open beaker. 0.1 ml aliquots were withdrawn at certain intervals and assayed as described above.

To study the effect of H_2 and CO on the air inactivation of the enzyme, experiments were carried out in stoppered 7 ml flasks. The flasks contained 2 ml hydrogenase solution (see above) and 5 ml gas mixtures consisting of 21% (v/v) O_2, a certain concentration of H_2 or CO and the corresponding amount of Ar at a total pressure of 101 kPa (760 mm Hg). Liquid samples were withdrawn with a hypodermic syringe for the assay.

Kinetics of oxygen inactivation of the enzyme

In aqueous solutions at pH 8.0 under the atmosphere of such gases as argon, nitrogen, or hydrogen, *C. pasteurianum* hydrogenase is stable for many hours. However, under air the enzyme rapidly and irreversible loses its catalytic activity (Fig. 1A, curve a). This inactivation is brought about by the oxygen in the air, inasmuch as under the gas mixture of 21% O_2 plus 79% Ar, the kinetic course of hydrogenase inactivation is the same as that under air (Fig. 1A, curve c). Therefore, in this paper we will use the terms oxygen inactivation and air inactivation interchangeably.

As shown in Fig. 1B, the time course of oxygen inactivation of the enzyme can be described in terms of first-order kinetics with the first-order rate constant $k = 0.015$/min. This corresponds to a half-life of approx. 46 min. It should be pointed out that different half-lives have been reported in the literature for oxygen inactivation of hydrogenase from *C. pasteurianum*. The values vary from 3—5 min [12,13,17] to 30—40 min and 2 h (Ballesteros, A., private communication). Such differences are seemingly due to variations in the con-

ditions of the growth of the bacterium and/or to use of different bacterial strains. Isolation and purification conditions may also affect the half-life.

Curve b in Fig. 1A represents the time course of oxygen inactivation of 5 mg/ml hydrogenase. Once can see that it is somewhat different from curve a (1 mg/ml hydrogenase). Further reduction in the enzyme concentration results in no appreciable change in the inactivation kinetics: upon 10-fold dilution of the enzyme (from 1 mg/ml—0.1 mg/ml) the rate constant changes (decreases) by less than 25%. One could speculate that at 5 mg/ml the inactivation process involves some contribution of polymolecular reactions (e.g., aggregation) or impurities in the enzyme preparation. However, at the lower concentrations (below 1 mg/ml) such processes play a negligible role. Therefore, all subsequent experiments were carried out with hydrogenase concentrations within the range from 1 mg/ml—0.1 mg/ml.

Results and Discussion

We have found that the natural electron carrier for hydrogenase from *C. pasteurianum*, ferredoxin from the same microorganism, greatly stabilizes the enzyme against irreversible oxygen inactivation (Fig. 2A). In the absence of ferredoxin the half-life of hydrogenase under air is 46 min, whereas in the presence of 57 μM ferredoxin it increases up to 11 h. The stabilization effect enhances with increase in the concentration of ferredoxin (Fig. 2A).

In contrast with ferredoxin, another electron carrier for the enzyme, FAD, drastically reduces the oxygen stability of the latter (Fig. 3A): 0.5 mM FAD increases the rate constant of air inactivation of hydrogenase by a factor of more than 5. The observed oxygen stability of hydrogenase decreases with the increase in the concentration of FAD (Fig. 3A).

The simplest kinetic scheme which can describe quantitatively the results obtained is the following:

$$\begin{array}{ccc} \mathrm{E} + \mathrm{X} & \overset{K}{\rightleftharpoons} & \mathrm{EX} \\ k \downarrow & & \downarrow \alpha \cdot k \\ \text{inactive hydrogenase} & & \end{array} \qquad (1)$$

where E is hydrogenase, X is an electron carrier, K is a binding constant, k is the rate constant of oxygen inactivation for the free enzyme, and α is a constant characteristic for a given electron carrier. When $\alpha < 1$, X stabilizes the enzyme, when $\alpha > 1$, X destabilizes the enzyme.

If the initial concentration of the enzyme is much lower than that of an electron carrier, $[\mathrm{E}]_0 << [\mathrm{X}]_0$, a mathematical treatment of scheme (1) yields the following equation:

$$\frac{k - k_{\mathrm{obs}}}{[\mathrm{X}]_0} = \frac{1}{K} \cdot k_{\mathrm{obs}} - \frac{\alpha \cdot k}{K} \qquad (2)$$

where k_{obs} is the observed rate constant of oxygen inactivation of the enzyme at a given concentration of X. Since k is known from independent experiments (in the absence of electron carriers, k = 0.015/min), by studying the depen-

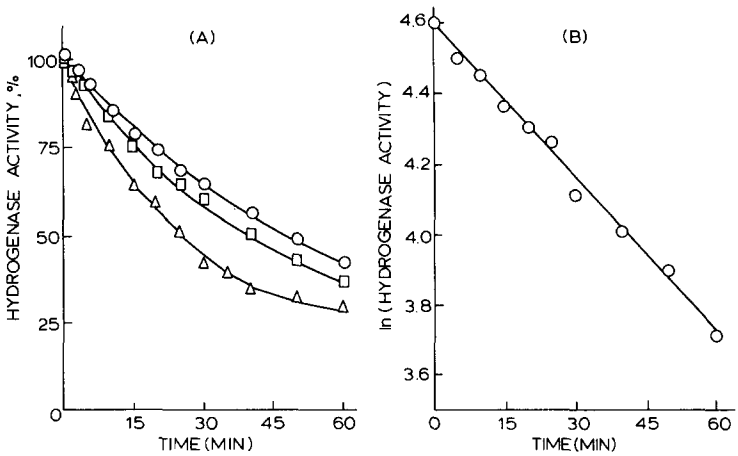

Fig. 1. Kinetics of oxygen inactivation of hydrogenase from *C. pasteurianum*. (A) 1 mg/ml hydrogenase under air (○——○) 5 mg/ml hydrogenase under air (△——△) 3 mg/ml hydrogenase under 21% O_2 + 97% Ar (□——□). (B) Linear presentation of curve (○——○) in semi-logarithmic coordinates. Conditions: 50 mM Tris-HCl buffer (pH 8.0), stirring at room temperature.

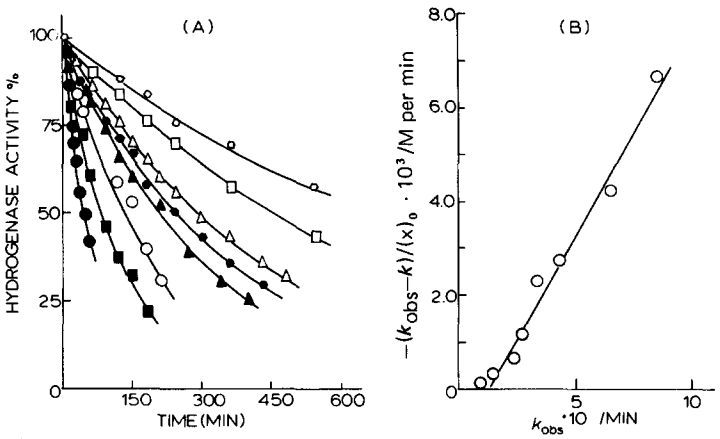

Fig. 2. Clostridial ferredoxin stabilization of *C. pasteurianum* hydrogenase against oxygen inactivation. (A) ●——●, in the absence of ferredoxin: in the presence of ferredoxin; ■——■, 0.57 μM; ○——○, 1.14 μM; ▲——▲, 2.85 μM; ●——●, 5.7 μM; △——△, 11.4 μM; □——□, 28.5 μM; ○——○, 57 μM. (B) Linear presentation of the data in Fig. 2A in accordance with Eqn. 2. Conditions: 50 mM Tris-HCl buffer (pH 8.0), 0.1 mg/ml hydrogenase, stirring under air at room temperature.

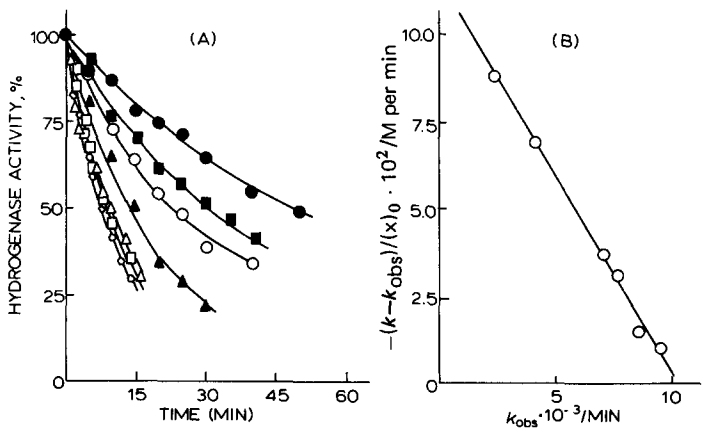

Fig. 3. FAD destabilization of *C. pasteurianum* hydrogenase against oxygen inactivation. (A) ●——●, in the absence of FAD: in the presence of FAD; ■——■, 10 μM; ○——○, 25 μM; ▲——▲, 50 μM; △——△, 100 μM; □——□, 200 μM; ○——○, 500 μM. (B) Linear presentation of the data in Fig. 3A in accordance with Eqn. 2. For experimental conditions see the legend to Fig. 2.

dence of k_{obs} on $[X]_0$, one can determine both α and K using Eqn. 2. As one can see in Figs. 2B and 3B, Eqn. 2 satisfactorily describes the effects of ferredoxin and FAD, respectively, on oxygen stability of hydrogenase.

We have used this approach to investigate quantitatively the effect of a number of electron carriers on the time course of oxygen inactivation of the enzyme (Table I). In most cases, experimental data fit Eqn. 2. In a few instances where they do not (see footnote to Table I), α and K were estimated numerically: the former using k_{obs} for saturating concentrations of X, the latter as [X] at which stabilization (destabilization) is a half-maximum.

As can be seen from Table I, such electron carriers as ferredoxin, methyl viologen and phenazine methosulfate greatly stabilize hydrogenase, while FMN, FAD and thionin drastically reduce its oxygen stability and others afford only a moderate effect. A significant feature of the data presented in Table I is that all electron carriers studied affect oxygen stability of hydrogenase even at very low concentrations which implies that stabilization/destabilization is quite specific.

It should be pointed out that some of the stabilizing electron carriers, e.g., NAD^+ and NADH, in fact fail to transfer electrons to/from the active center of the enzyme at a measurable rate [18]. Our data indicate that both NAD^+ and NADH bind to the active center of hydrogenase. The redox potential for NAD^+ is between those of methyl viologen and FAD and FMN which are very effective electron carriers for *C. pasteurianum* hydrogenase-catalyzed oxidation of molecular hydrogen [18]. Hence the inability of NAD^+ to mediate this reaction is apparently due to its incorrect orientation at the active center of the enzyme.

In order to understand why some electron carriers stabilize hydrogenase whereas others destabilize it, we carried out some competitive experiments. The effect of benzyl viologen (which only slightly affects the oxygen stability

TABLE I

VALUES OF α AND K FOR THE EFFECT OF VARIOUS ELECTRON CARRIERS ON OXYGEN STABILITY OF HYDROGENASE FROM *CLOSTRIDIUM PASTEURIANUM*

$\alpha < 1$ corresponds to stabilization of the enzyme, $\alpha > 1$ corresponds to destabilization (Eq. 1). See Material and Methods for experimental conditions.

Electron carrier, X	α	K (μM)
C. pasteurianum ferredoxin	0.06	0.74
Methyl viologen	0.07	910
Benzyl viologen	0.70	—
Polybutenyl viologen	0.28	80
Methylene blue	0.50 *	100—200 *
FMN	11	14
FAD	7	96
2,6-Dichlorophenol-indophenol	0.23 *	2.5—5.0 *
Phenazine methosulfate	0.08	0.5
Neutral red	0.25	1.2
Thionin	6 *	50 *
NAD^+	0.40	<50
NADH	0.14	700

* Experimental data did not fit Eqn. 2 satisfactorily. Therefore, the values of α and K shown above were calculated as follows: the former by using k_{obs} for saturating concentration of X, the latter as the concentration of X at which stabilization (destabilization) was half-maximum.

of the enzyme) was studied on the stabilization brought about by methyl viologen. As one can see in Fig. 4 (curve a), increasing concentrations of benzyl viologen progressively reduce stabilization of the enzyme by methyl viologen. At concentrations of benzyl viologen higher than 100 μM, k_{obs} is equal to that in the presence of benzyl viologen alone. These data unambiguously indicate that methyl viologen and benzyl viologen compete for the binding with the enzyme, that is, they bind either to the same subsite of the active center or to overlapping subsites. In contrast with the methyl viologen results, benzyl viologen does not affect the degree of hydrogenase destabilization brought about by FMN (Fig. 4, curve b). This clearly indicates that FMN and benzyl viologen (and hence methyl viologen) bind to different and independent subsites of the active center of the enzyme.

Why do some electron carriers stabilize the enzyme while others destabilize it? Also, what is the basis for the relative magnitude of their effect? We have failed to find any correlation between α and K on one hand and such parameters as the redox potential, electrostatic charge or chemical structure on the other hand. Hence, these questions remain.

Molecular hydrogen is a natural substrate for hydrogenases and carbon monoxide is a potent reversible inhibitor of the enzyme [10,11]. Therefore, it was of particular interest to study their effect on oxygen inactivation of the enzyme. We have studied the effect of increasing concentrations of both H_2 and CO on the time course of oxygen inactivation of clostridial hydrogenase. As one can see in Fig. 5, neither of the gases changes appreciably the rate constant of this inactivation despite the fact that maximal concentrations of the gases used were close (in the case of H_2) or exceeded by far (in the case of CO)

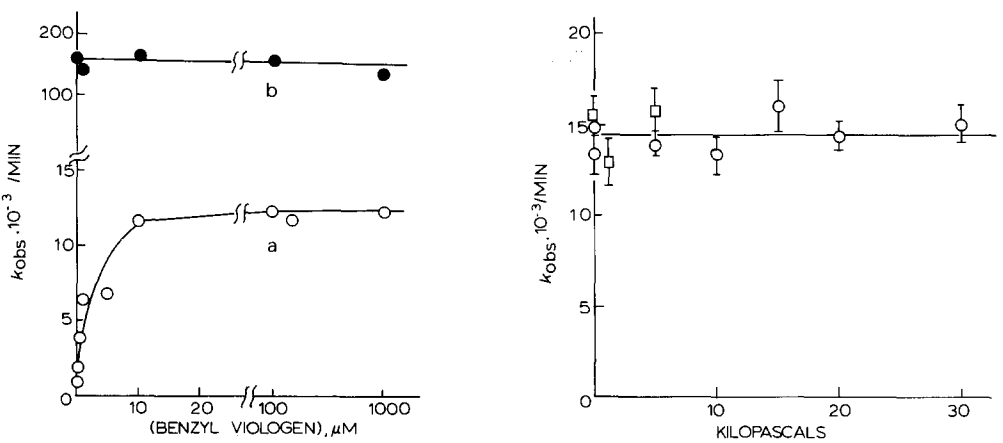

Fig. 4. The effect of benzyl viologen on stabilization of *C. pasteurianum* hydrogenase by 10 mM methyl viologen (○———○) and on destabilization of the enzyme by 100 μM FMN (●———●). For experimental conditions see the legend to Fig. 2.

Fig. 5. The effect of H_2 (○———○) and CO (□———□) on oxygen inactivation of hydrogenase from *C. pasteurianum*. Hydrogenase solutions (1 mg/ml) were incubated at room temperature with stirring in serum stoppered flasks containing 21 kPa of O_2, a certain concentration of H_2 or CO and the corresponding concentration of Ar to bring the total pressure to 101 kPa (760 mg Hg). Tris-HCl buffer (pH 8.0).

the binding constants with the enzyme determined in kinetic experiment, i.e., 40 and 0.666 kPa correspondingly [18].

H_2 and CO compete for the same binding subsite in the active center of hydrogenase from *C. pasteurianum* [18]. This subsite is apparently an iron atom of the iron-sulfur cluster of the active center of the enzyme [10]. On the other hand, CO, although a competitive inhibitor of the enzyme with respect to H_2, is a noncompetitive inhibitor vs. methyl viologen [18]. Hence, the binding subsite for CO and H_2 is different from that for methyl viologen.

Therefore, on the basis of our results reported here and kinetic data obtained by Erbes and Burris [18], one can propose that the active center of *C. pasteurianum* hydrogenase consists of at least three independent subsites (Fig. 6). The first one (probably, an iron atom of the iron-sulfur cluster) binds H_2 and CO. The second one binds methyl viologen and other stabilizers (Table I). The third one binds FMN, FAD and thionin. It is known from our previous experiments [13] that oxygen inactivation of clostridial hydrogenase involves the oxidation of the mercaptide-sulfide constituents of the iron-sulfur clusters. Hence, one could speculate that whereas binding to subsite I (Fig. 6) does not affect the rate of this oxidation, binding to subsite II (Fig. 6) shields the oxidizable groups from molecular oxygen from solution, while binding to subsite III (Fig. 6) induces a conformational change which results in a greater exposure of those groups to oxygen.

It should be mentioned that the above described properties of hydrogenase from *C. pasteurianum* are markedly different from those of the enzyme from other sources. For example, Adams and Hall [19] have found that benzyl viologen greatly protects *Escherichia coli* hydrogenase against O_2 inactivation. In the case of clostridial hydrogenase this stabilization is insignificant (Table I). In another interesting study [20], it has been found that CO stabilizes hydrogenase from *Chlamydomonas reinhardi* against O_2 inactivation. As one can see from our work, the rate of oxygen inactivation of *C. pasteurianum* hydrogenase is not affected by carbon monoxide (Fig. 5).

Our findings have important biotechnological implications. Suppose stabilized (e.g. Refs. 12, 13) *C. pasteurianum* hydrogenase (which is one of the most catalytically active hydrogenases known) is to be used in practice. From

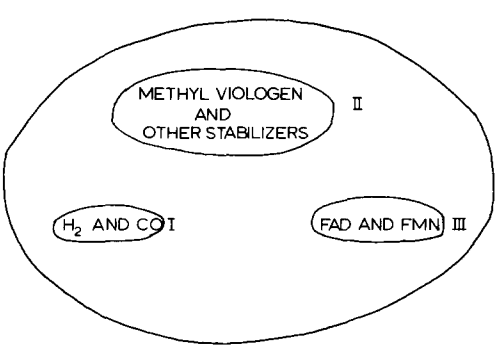

Fig. 6. Schematic representation of the active center of hydrogenase from *C. pasteurianum*. Subsite I (possibly an iron atom of the iron-sulfur cluster) binds H_2 and CO. Subsite II binds methyl viologen and other stabilizers from Table I. Subsite III binds FMN and FAD.

the standpoint of their capability of transferring electrons one can choose a number of different electron carriers, e.g., methyl viologen or FMN. The rates of the hydrogenase-catalyzed oxidation of molecular hydrogen mediated by these compounds are very close [18]. However, while methyl viologen is an effective stabilizer of the enzyme, FMN drastically destabilizes hydrogenase. Therefore, the use of the former electron carrier is obviously indicated. In general, when one employs electron carriers for an enzyme for practical use, the effect of electron carriers on stability of the enzyme must be known and appropriately considered.

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