IMMOBILIZATION OF HYDROGENASE ON GLASS BEADS

by

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SUMMARY

Hydrogenase from Clostridium pasteurianum was immobilized on glass beads by four different methods. The sensitivity of the native and bound enzyme to oxygen was examined. Hydrogenase bound to succinyl glass proved to be the most stable to oxygen. All bound enzymes were active with ferredoxin as a substrate and evolved hydrogen in a chloroplast-ferredoxin-hydrogenase system driven by light.

Hydrogenase from Clostridium when coupled to the photosynthetic system can produce hydrogen gas with water as the electron donor (1). This system has been proposed as a basis for utilization of solar energy. It has also been shown that hydrogen evolution in a chloroplast-ferredoxin-hydrogenase system is enhanced by addition of a mixture composed of glucose, glucose oxidase and catalase presumably active as an oxygen scavenger. Inhibition of the water-splitting photosystem II by 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, using electrons donated from ascorbate-reduced 2,6-dichlorophenolindophenol, also enhances hydrogen evolution (1). The well-known hydrogenase sensitivity to oxygen (2) reflected in these findings poses a problem for long-term hydrogen evolution using the Clostridium hydrogenase.

Immobilization of an enzyme on glass beads can stabilize it over wide ranges of pH and heat. Different methods of binding give differing stabilities (3). We have examined the oxygen sensitivity of Clostridium pasteurianum hydrogenase and bound the enzyme to glass using several methods. Our results indicate that oxygen sensitivity of the immobilized enzyme is greatly reduced.

MATERIALS AND METHODS

C. pasteurianum ferredoxin was purified by the method of Mortenson (4). Partial purification of hydrogenase was accomplished according to Nakos and Mortenson (5) with the omission of the last Sephadex column treatment. The enzyme assay was that of Benemann et al. (1). Except as noted, the assay mixture contained 1 µmole of methyl viologen, 15 µmoles of sodium dithionite and 50 µmoles of TRIS, pH 8.0. Synthesis of alkylamine glass was done by the method of Weetall and Filbert (6), that of succinyl glass was as described by Weetall (7).

Binding methods are illustrated in Fig. 1. Sixty mg of 1-ethyl-3-(3-dimethyl-amino-propyl-carbodiimide HCl) (purchased from Sigma) and 1 ml of 15-30 mg/ml protein solution was added to 0.5 gm succinyl or alkylamine beads in 2 ml 0.1 M PO_4, pH 7.0, in a stoppered 5 ml Fernbach flask under hydrogen. The mixture was shaken at 4°C for 3 hours at which time the solution became slightly turbid. The beads were then filtered under a stream of hydrogen gas and washed with 500 ml of 1 M NaCl and 500 ml of 0.1 M PO_4 buffer. Both solutions had been bubbled with hydrogen and 1 mM sodium dithionite was added to remove all oxygen. These products are referred to as "succinyl-bound hydrogenase" or "alkylamine-bound hydrogenase," respectively. Glutaraldehyde-bound hydrogenase was prepared by adding 3 ml of 5% glutaraldehyde v/v in 0.1 M PO_4, pH 7.0, to 5 gm alkylamine beads. The mixture was evacuated in a dessicator for 1 hour. The beads were then washed with 500 ml distilled water and placed in a stoppered Fernbach flask under hydrogen. Two ml 0.1 M PO_4, pH 7.0, and 1 ml of a 15-30 mg/ml protein solution were added. The mixture was allowed to shake overnight and filtered the next morning as before.

Arylamine glass was diazotized by adding 10 mls of 2 N HCl and 0.25 gm sodium nitrite to 0.5 gm alkylamine glass beads and placing in a dessicator which was then evacuated. After an hour, the beads were washed with 500 ml 0.1 M PO_4 buffer, pH 7.0. Two ml 0.1 M PO_4, pH 7.0, buffer was added and the mixture placed under hydrogen. One ml of a 15-30 mg/ml protein solution was then added and shaken at 4°C overnight. The beads were then filtered in the same manner

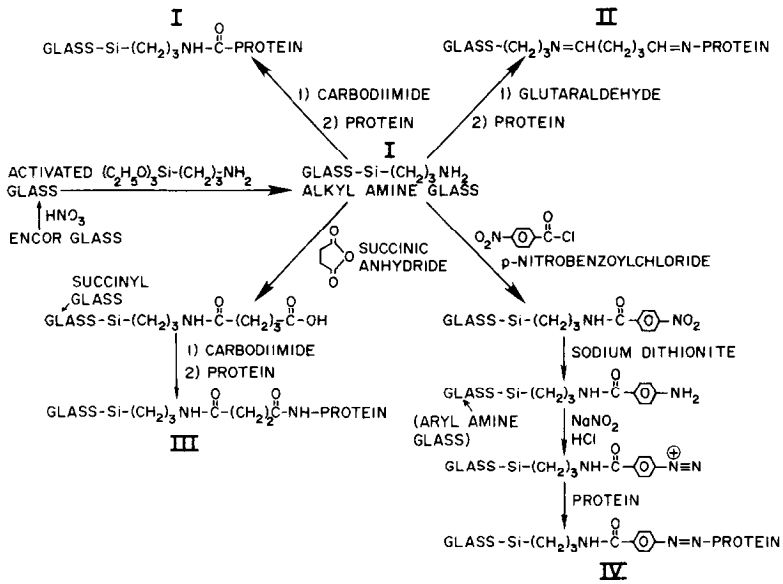

Figure 1. Methods of binding hydrogenase to glass beads.

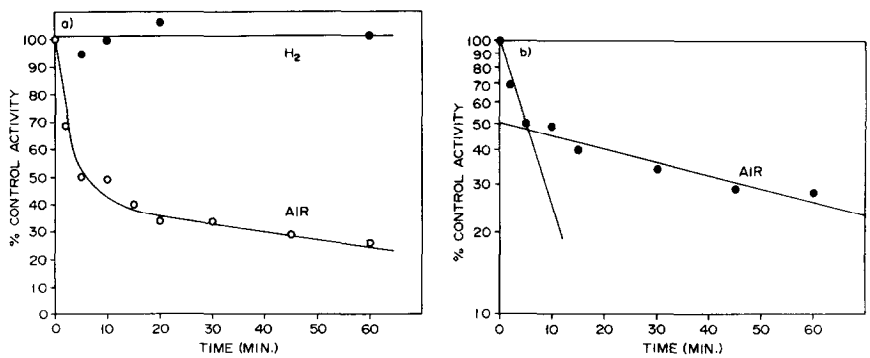

Figure 2a. Inactivation of soluble hydrogenase as a function of time exposed to air. Enzyme stored under H_2 was diluted 10-fold into air equilibrated buffer under air. At intervals, aliquots were removed and assayed according to the procedure in the Materials and Methods section. Controls were kept under H_2.

Figure 2b. Inactivation of soluble hydrogenase plotted logarithmically vs. time exposed to air.

as the alkylamine-carbodiimide beads. This product is referred to as "diazo-bound hydrogenase." Protein analysis was by the method of Böhlen et al. (8). To determine protein remaining in solution in binding experiments, samples were removed before filtering.

TABLE 1. Binding of Hydrogenase to Glass Beads

Type of Linkage	A Protein Added (mg)	B Activity Added μmoles H_2 Produced min	C Protein Unbound	D Total Activity Bound	$\overrightarrow{D}$ A-C Specific Activity
diazo	20	420	<0.4	12.6	0.63
glutaraldehyde	20	420	<0.4	26.0	1.3
alkylamine-carbodiimide	20	420	3.3	8.5	0.51
succinyl-carbodiimide	20	420	11.4	11.1	1.3

RESULTS AND DISCUSSION

Figure 2a shows the inactivation of hydrogenase as a function of time exposed to air. Figure 2b shows the same data with the ordinate axis plotted logarithmically. This plot reveals two slopes, indicating two rates of inactivation. This would imply two mechanisms of inactivation, a two-step mechanism or two different enzyme states which have differing sensitivities. Nakos and Mortenson have presented evidence against the existence of isoenzymes of differing charge (9).

Table 1 shows the results of a representative binding experiment. Protein binding was virtually complete in the case of diazo and glutaraldehyde linkages, but not with alkylamine binding with carbodiimide (16% of the protein remained unbound) nor succinyl glass (43% of the protein unbound). It should be noted that these percentage figures are minimal for unbound protein, because there may be traces of protein bound noncovalently. Thus, the specific activity values of Table 1 are minimal. To remove the possibility of noncovalently bound protein, the beads were washed with 1 M NaCl. Activity measurements were made using methyl viologen as the electron donor.

While protein binding was above 50%, activity on the glass was as low as 2.0% (for alkylamine-carbodiimide binding) and only as high as 5.2% (for gluta-

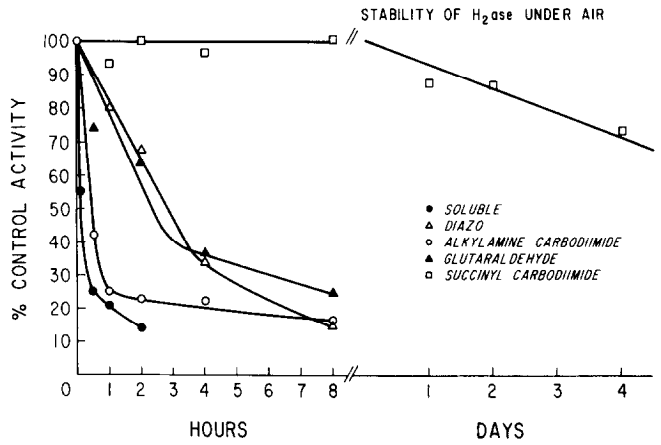

Figure 3. Inactivation of bound hydrogenase vs. time exposed to air. Assay mixtures contained 1 ml final volume: 50 µmoles Tris, pH 8.0, 1 µmole methyl viologen, 15 µmoles sodium dithionite and 30-50 mg glass beads were placed in Fernbach flask in buffer under argon and at time 0 exposed to air. Individual flasks were assayed at indicated times.

raldehyde binding). Low recoveries of activity after immobilization have been reported previously (10). While succinyl glass bound the least protein, the specific activity of these beads was comparable to the glutaraldehyde glass.

Figure 3 shows the sensitivity of these glass-bound enzymes and the native enzyme to aerobic conditions. All four forms of binding stabilize the enzyme to oxygen relative to the native enzyme. Succinyl-bound hydrogenase is the most stable form for bound Clostridial hydrogenase increasing the time for loss of 50% of the activity from 1-2 minutes for the native enzyme to several days. The biphasic nature of the oxygen inactivation of the soluble enzyme is retained in at least three types of binding. This indicates the mechanism of oxygen inactivation is retained, but that at least the initial rate has been retarded.

Table 2 shows that glass bound hydrogenases are able to use Clostridial ferredoxin, reduced by dithionite, as a substrate. However, the specific activity, normalized per gram of glass beads, varies over a 20-fold range. This is in contrast to the activities using methyl viologen which vary only over a 3-fold range. The size of the ferredoxin, with a molecular weight of 5,600 (4), may hinder its penetration onto the pores of the glass. Methyl

TABLE 2. Hydrogen Evolution with Glass-Bound Hydrogenase

Type of Bound Hydrogenase	Dithionite[a] μmoles/min g glass bead	Activity with Ferredoxin / activity with methyl viologen
diazo	0.12	0.022
alkylamine	1.9	0.37
succinyl	0.097	0.016
glutaraldehyde	1.0	0.096

[a]Dithionite assays contained in a total volume of 1 ml 15 μmoles of sodium dithionite, 45 μg C. pasteurianum ferredoxin, 15 μmoles of HEPES (pH 7.6), 1.5 μmoles of $MgCl_2$ and 30-50 mg of glass bound hydrogenase. Reaction time was 15 min. Reactions were terminated and measured according to ref. (1).

viologen, with a molecular weight of 257, is not so restricted by the glass and may more easily penetrate the pores. In the case of soluble hydrogenase, the ratio of hydrogen produced with ferredoxin as a substrate to hydrogen produced using methyl viologen as a substrate is 0.39. The ratios of the various glass bound enzymes are shown in Table 2. This parameter is an indicator of the relative abilities of the glass bound enzyme to utilize ferredoxin as a substrate. No glass bound enzyme has a greater ratio than the soluble enzyme, though alkylamine bound hydrogenase with a ratio of 0.37 is similar to the soluble enzyme. This type of binding also yields the product most sensitive to oxygen. In fact, these ratios are ordered in a manner opposite to the oxygen sensitivity. The lowest ratio is for succinyl bound hydrogenase, which is the least oxygen sensitive. These data suggest that the same factors which reduce oxygen sensitivity may play a role in reducing the ability of the enzyme to utilize ferredoxin as a substrate. We plan to explore this possibility by using poreless styrene beads and soluble dextrins as a matrix for binding.

All glass bound hydrogenases are able to produce hydrogen using ferredoxin reduced by illuminated chloroplasts. As a control, alkylamine glass, with no

enzyme bound to it, produces no hydrogen. These results indicate hydrogenase bound to glass beads can be used to produce hydrogenase in a chloroplast-ferredoxin-hydrogenase system driven by light. Furthermore, glass bound hydrogenase has the advantage of being less sensitive to oxygen. Further work must be done to find optimal conditions for hydrogen production such as type and concentration of ferredoxin, and whether other solid supports will overcome some of the disadvantages of glass.

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1. Benemann, J.R., Berenson, J.A., Kaplan, N.O., and Kamen, M.D. (1973) Proc. Nat. Acad. Sci, USA 70, 2317-2320. See also Krampitz, L.O. (1972) An inquiry into biological energy conversion, A. Hollaender et al., Eds. A report on a workshop at Gatlinburgh, Tenn., October, 1972, p. 22. University of Tennessee, Knoxville, Tennessee.

2. Mortenson, L.E. and Chen, J.-S. (1974) in Microbial Iron Metabolism, J.B. Neilands, Ed., Academic Press, pp. 231-282.

3. Dixon, J.E., Stolzenbach, F.E., Lee, C.T., and Kaplan, N.O. (1974) Israel J. Biochem. 12, 529-541.

4. Mortenson, L.E. (1964) Biochim. Biophys. Acta 81, 71-77.

5. Nakos, G. and Mortenson, L.E. (1971) Biochim. Biophys. Acta 227, 576-583.

6. Weetall, H.H. and Filbert, A.M. (1974) in Methods in Enzymology, Vol. XXXIV, W.B. Jakoby and M. Wilchek, Eds., Academic Press, pp. 59-72.

7. Obtainable from H.H. Weetall, Corning Glass Works, Corning, N.Y.

8. Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220.

9. Nakos, G. and Mortenson, L.E. (1971) Biochem. 10, 2442-2449.

10. Lartigue, D.J. (1975) in Immobilized Enzymes for Industrial Reactors, R.A. Messing, Ed., Academic Press, pp. 125-135.