IMMOBILIZATION OF HYDROGENASE AND FERREDOXINS ON GLASS BEADS

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1. Introduction

Lappi et al. [1] have recently demonstrated that *Clostridium pasteurianum* hydrogenase immobilized on glass beads by a variety of methods evolves hydrogen when using dithionite as reducing agent and methyl viologen or ferredoxin as electron carrier. The immobilized enzyme preparations were more resistant to oxygen inactivation than the soluble enzyme. We obtained similar results and report that ferredoxins also may be immobilized on glass beads. The immobilized hydrogenase can utilize soluble ferredoxin reduced by illuminated chloroplasts as an electron donor and immobilized ferredoxin can itself be reduced by illuminated chloroplasts.

2. Materials and methods

Spinach ferredoxin was prepared by a method modified after Tagawa and Arnon [2]. Hydrogenase and ferredoxin from *C. pasteurianum* and chloroplast grana were prepared and hydrogen was assayed as described previously [3]. The reactions, carried out at 23°C for 15 min, contained in 1 ml final volume: 15 μmol HEPES buffer (pH 7.6 with 1.5 μmol $MgCl_2$) hydrogenase, ferredoxin as indicated in the legends and to start the reaction, an electron donor solution using dithionite (20 μmol $Na_2S_2O_4$) water (a chloroplast preparation containing 300 μg chlorophyll) or ascorbate (same amount of chloroplasts and 10 μmol sodium ascorbate, 0.05 mol 2,6-dichlorophenolindophenol and 0.1 μmol of 3-(3,4-dichlorophenyl)1,1-dimethylurea). 15–40 mg of hydrogenase and ferredoxins were bound per gram of 550 Å alkylamine or carbodiimide activated glass beads under hydrogen using procedures previously described [1,4,5].

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3. Results and discussion

Table 1 shows that *C. pasteurianum* hydrogenase could be immobilized in an active form on glass beads. The immobilized hydrogenase preparations were catalytically active with the electron donor–electron carrier system dithionite–ferredoxin. Extensive washing of the beads or treatment with 1% albumin, known to remove noncovalently bound protein, resulted in losses of activity up to 50%. In table 1 all data except for experiment 2 column 4 refers to hydrogenase activity after albumin treatment. Upon immobilization the specific activity of the hydrogenase preparation normally decreased by more than 90% of the original activity. This loss of specific activity could be due to denaturation of bound enzyme and/or stearic effects. The greater importance of stearic effects is indicated by the much higher specific activity when using the smaller electron carrier methyl viologen compared to ferredoxin.

The bound hydrogenase preparations were more resistant than the soluble hydrogenase toward oxygen inactivation. After one hour under 100% O_2, less than half the activity of the bound enzyme was lost. Soluble hydrogenase lost about 80% of its activity in 10 min under air. The remaining soluble enzyme activity inactivated at a much slower rate. This indicates that

Table 1
Activities of *C. pasteurianum* hydrogenase with dithionite before and after immobilization on glass

Experiment	Ferredoxin in assays (nmoles)	Soluble		Immobilized with carbodiimide		Immobilized with glutaraldehyde	
		nmoles H_2/15 min	spec. act.	nmoles H_2/15 min	spec. act.	nmoles H_2/15 min	spec. act.
1	16	4400	1.4	180	0.016	105	0.009
2	25	2842	1.5	611	0.25	720	0.06
3	5	3042	3.0	528	n.d.	290	0.02

Spec. act. in μmol H_2/min/mg hydrogenase.

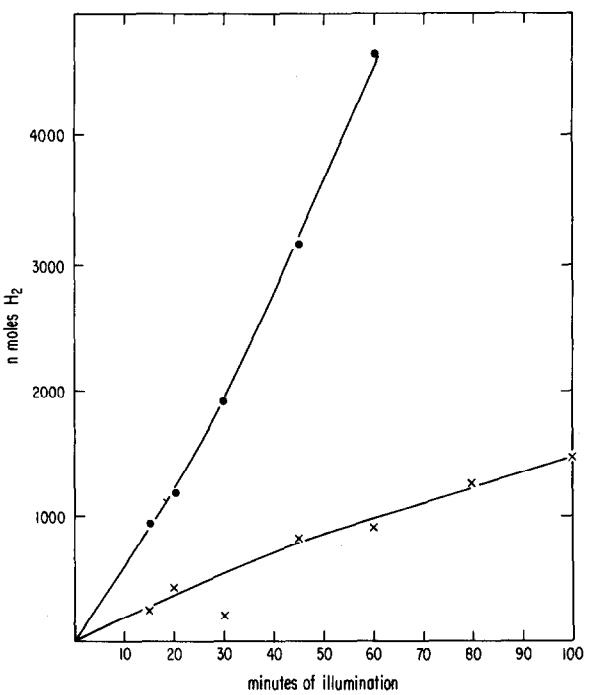

Fig.1. Time course for glutaraldehyde bound *C. pasteurianum* hydrogenase activity in the chloroplast assays. Assays (see Materials and methods) contained 16 nmol soluble *C. pasteurianum* ferredoxin and 1.7 mg *C. pasteurianum* hydrogenase bound to glass beads (54 mg). Hydrogenase activity was determined by withdrawing 1 ml gas samples at staggered intervals from duplicate assays after injecting 1 ml argon (data corrected for gas dilution). Electron donor: water (–X—X–); ascorbate (–•—•–).

a relatively oxygen stable hydrogenase fraction exists which could account for the oxygen stability of the small amount of active hydrogenase immobilized.

The immobilized hydrogenases are active using either water or ascorbate as electron donors in the chloroplast–ferredoxin hydrogenase system [3] (fig.1). Reactions are linear for at least one hour. The ratios between water and ascorbate driven hydrogen production is similar to that of the soluble enzyme [3].

Table 2 shows the results of binding *C. pasteurianum* and spinach ferredoxins to alkylamine glass via glutaral-

Table 2
Activities of soluble and immobilized ferredoxins assayed with soluble hydrogenase and dithionite or ascorbate as electron donors

Experiment	Ferredoxin origin	Soluble ferredoxin			Glutaraldehyde bound ferredoxin		
		Ferredoxin (nmoles)	Dithionite assay	Ascorbate assay	Ferredoxin (nmoles)	Dithionite assay	Ascorbate assay
1	*C.pasteurianum*	16	431	88	19	105	12
2	*C.pasteurianum*	10	108	135	32	16	11
3	Spinach	17	193	62	56	35	1.4

Activities expressed as nmoles H_2 evolved in 15 min/nmol ferredoxin. Hydrogenase in the dithionite assays was 2.5 μg for experiments 1 and 3 and 100 μg for experiment 2. Ascorbate assays contained about twice as much hydrogenase.

dehyde. In the case of spinach ferredoxin all the protein (at 20 mg/g glass bead) was bound, resulting in a colorless supernatant and a deep-red glass bead preparation. The complete binding and high specific activity observed for this protein demonstrates that iron–sulfur proteins can be immobilized with a high degree of effectiveness. An active ferredoxin bound to glass beads using carbodiimide could not be demonstrated. The immobilized electron carriers are able to transfer electrons from sodium dithionite or ascorbate (via illuminated chloroplasts) to soluble hydrogenase (table 2). The immobilized ferredoxins are 5–20% as active (on a nmol basis) as the soluble ferredoxins when assayed using soluble hydrogenase and sodium dithionite. The ratio of ascorbate to sodium dithionite activities for the bound ferredoxins is much smaller than for soluble ferredoxins, again suggesting stearic effects. Mixing of the immobilized hydrogenase and ferredoxins, or binding on the same glass beads, gave no activity in any assay.

The utilization of immobilized enzyme systems in solar energy conversion, as suggested by Lappi et al. [1] and implied by fig.1, is not a plausible undertaking. Hydrogenase resistence to oxygen inactivation might not be a result of the immobilization process and, at any rate, highly stable and oxygen resistant soluble hydrogenases are known [6].

Since the product is volatile, immobilized hydrogenases have no apparent advantage over the soluble enzyme in the chloroplast–ferredoxin hydrogenase system. (fig.1). The lack of demonstrated oxygen evolution by this system [3,7] makes it uncertain for biophotolysis applications. Algae based biophotolysis systems are theoretically [8–10] no less efficient and economically more viable, than biochemical ones. One significant and more feasible application of immobilized hydrogenase (and ferredoxins) is in the development of inexpensive biochemical hydrogen–oxygen fuel cells. This would require immobilization of iron–sulfur proteins on an electrode such that electron-transfer is possible. Biochemical fuel cells could significantly improve the prospects of biophotolysis by increasing several-fold the useful work that can ultimately be derived from photosynthetic hydrogen production.

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