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Immobilization of Isolated and Cellular Hydrogenase of *D. desulfuricans* in Radiation-Polymerized Polyacrylamides

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Abstract

Purified hydrogenase from *Desulfovibrio desulfuricans* was immobilized either by entrapment or absorption onto porous neutral and charged acrylamide beads. Surface absorption and crosslinking on the beads resulted in a high hydrogenase activity and a good immobilization coefficient compared to the enzyme and whole cells entrapped in the same matrix. Maximum enzyme activity (citrate–phosphate buffer) was shifted to pH 6.5 upon immobilization in contrast to 6.0 for the free enzyme and the range of 6–7 for whole cells. Both the purified enzyme and whole cells were most active when held in neutral matrices. Immobilization improved the temperature stability (65°C) and long term storage (4°C) of the hydrogenase activity of both the purified enzyme and whole cells.

Index Entries: Hydrogenase, immobilized; *Desulfovibrio desulfuricans*, purified hydrogenase from; enzyme immobilization; radiation-polymerization; polyacrylamide beads; immobilized hydrogenase;

Introduction

Hydrogenase (E.C. 1.12.2.1) is contained in many anaerobic hydrogen-metabolizing microbes (*1–3*). The enzyme catalyzes the following reaction:

$$2H^+ + 2e \rightleftarrows H_2$$

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The two electron driving force for the hydrogen production direction can be supplied by chemical or photochemical means. Indeed, the enzyme has been applied to various chemical and photochemical systems to either use or produce hydrogen gas (*3–6*). Difficulty in obtaining adequate quantities of hydrogenase hinders further studies in this area. To ameliorate this situation, a more amenable organism producing large quantities of the enzyme or a system to reuse the available quantities of the enzyme is required. In similar circumstances, immobilization has been suggested as a general method by which enzyme reuse may be made possible (*5–9*).

Previous studies on the immobilization of the hydrogenase activity of the sulfate reducing anaerobe *Desulfovibrio desulfuricans* dealt with entrapment of the hydrogenase activity within the cells of the microorganism by glutaraldehyde treatment (*9*). Now we describe the immobilization of the purified enzyme of *D. desulfuricans* and compare its properties with entrapped cell-associated hydrogenase. The purified enzyme was immobilized in several different ways. The activity (immobilization coefficient), matrix charge effects, pH profile, as well as thermal and oxygen stabilities of the immobilized enzyme were determined. Long-term stability of the various immobilized enzyme preparations were also examined.

Materials and Methods

Sterile filtered water was deionized before use. All chemicals used to prepare buffers were reagent grade and used as received. Acrylamide, acrylic acid, and methylene-bis-acrylamide were obtained from commercial sources. MAPTAC (methacrylamide–propyltrimethylammonium chloride) and DMAPMA (dimethylaminopropyl–methacrylamide) were obtained as gifts from Texaco, Bellaire, Texas. Bovine serum albumin was obtained from Sigma Chemical, St. Louis MO. Glutaraldehyde (50% w/v, Fisher Scientific) was used as received without purification.

Whole Cell Immobilization

Desulfovibrio desulfuricans (NRC 49001) was grown (*10*) and hydrogenase activity entrapped by glutaraldehyde treatment (phosphate buffer) as previously described (*9*). Whole cells, 2% w/v in the polymerizing mixture, were immobilized in various matrices as previously described (*9*) (cf., entrapment immobilization below).

Purified Enzyme Immobilization

Hydrogenase from *D. desulfuricans* was purified (to the Sephacryl step) as reported before (*11*). Specific activity of the preparations varied between 6000 and 9000 μmol H_2/min/mg.

1. Entrapment immobilization. Beads (3 mm diameter) containing purified hydrogenase were prepared by the low temperature, ^{60}Co-radiation polymerization technique (*9,12*). The polymerization mixture in 0.05*M* potassium phosphate, pH 7.5 buffer had the following composition: either 26.4% w/v acrylamide or 13.2% w/v acrylamide plus 13.2% w/v comonomer, methylene-bis-acrylamide 3.3% w/v, tetraethylmethylenediamine (TEMED) 0.05% w/v, bovine serum albumin (BSA) 1 mg/mL, and purified hydrogenase 0.0007 mg/mL.

2. Absorption immobilization. Beads (3 mm diameter) were prepared by the low-temperature, ^{60}Co-radiation technique. The polymerization mixture contained 13.2% w/v acrylamide, 13.2% w/v acrylic acid, and 3.3% w/v methylene-bis-acrylamide in water at pH 7. This was extruded into *n*-pentane at −70°C and irradiated (^{60}Co source, 460 krad total delivered dose) as previously described (*9*). Beads were cured *in vacuo* overnight, washed with water, 0.01*N* HCl, water to neutrality, and lyophilyzed. Hydrogenase was linked to the presoaked beads by incubation of the enzyme, for 4 h at 25°C, in 0.1*M* sodium acetate buffer, pH 5.0, containing 0.2% w/v bovine serum albumin. Enzyme activity was prevented from leaching by glutaraldehyde treatment (0.1% w/v in water, 2 h). The beads were then washed well before use with a suitable buffer (Fig. 1, Table 4).

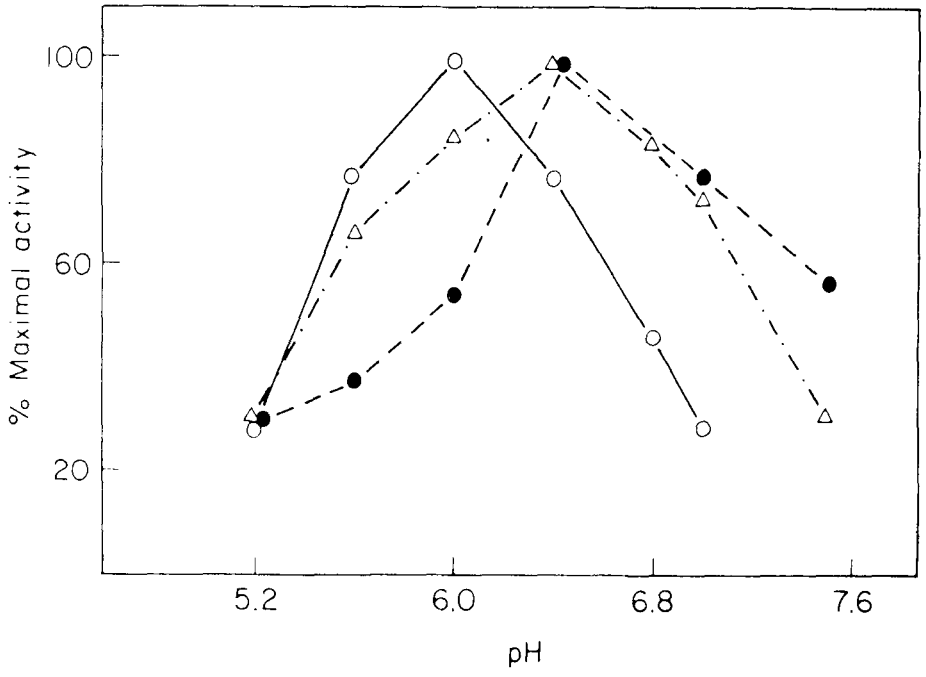

Fig. 1. Relative activity of various hydrogenase preparations vs pH. Purified hydrogenase (0—0); *D. desulfuricans* cells (Δ); immobilized hydrogenase (absorption) (●— —●). Experimental conditions: All buffers used were 0.2*M* citrate–phosphate (McIlvaine's buffer). Enzyme preparations had the following specific activities measured at pH 5.5 (acetate, 0.1*M*): purified enzyme, 6500 U/mg protein; *D. desulfuricans* cells, 14 U/mg protein; absorbed hydrogenase–BSA, 1500 U/mg total protein bound.

Hydrogenase Activity Measurements

Hydrogen evolution measurements were made using a modified oxygen electrode (*13*). Activities were measured routinely at pH 5.5 (acetate) for the free enzyme (*9*) and at pH 6.5 (phosphate) for the whole cells (*11*).

Results and Discussion

The immobilization and behavior of the cell-associated hydrogenase of *Desulfovibrio desulfuricans* was previously described (*9*). Because enzymes can have different properties when isolated from their natural environment, we wished to examine the immobilization and behavior of the *D. desulfuricans* hydrogenase after purification. To this end, the purified hydrogenase was immobilized either by entrapment or by an absorption and crosslinking procedure. One striking observation is that the isolated enzyme is relatively unstable at the low concentrations usually required for the assay method. Therefore, bovine serum albumin was used in all solutions (1 mg/mL) to prevent deactivation by absorption on the glass surfaces and to extend the useful lifetime of the enzyme.

Immobilization of the hydrogenase by entrapment in the interior of polyacrylamide beads resulted in moderate activity, but a low immobilization coefficient (Table 1). These effects were probably caused by hindered access to substrate inside the beads.

Absorbing the protein on a porous ion exchange beads resulted in preparations with high hydrogenase activity and with good immobilization coefficients. The incorporation of BSA into the solution used for absorption not only improved the

TABLE 1
Activity and Immobilization Coefficients of Hydrogenase Entrapped or Absorbed on Polyacrylic–Polyacrylamide Beads[a]

	Specific activities[b]		
Immobilization technique	Units per bead	Hydrogenase units per mg of total protein immobilized	Immobilization[c] coefficient, %
Entrapment	0.022	1.8	6.5
Absorption			
Surface bound − BSA	0.13	77.4	3.8
Surface bound + BSA[d]	0.04	0.67	18
Entrapped whole cells	0.03	0.44[e]	9

[a]Radiation-polymerized beads (3 mm diameter) were prepared as per experimental.
[b]Activities were measured at pH 5.5 for the enzyme and at pH 6.5 for whole cells.
[c]Immobilization coefficients were calculated from the equation $A_B/A_S - A_F$ where A_B = total activity measured for beads, A_S = total activity in solution before immobilization, A_F = total activity in solution after immobilization.
[d]Enzyme solutions were stabilized by BSA (1 mg/mL polymer mixture).
[e]No excess protein remained after entrapment.

TABLE 2
Matric Charge Effects on the Relative Activity of Immobilized Hydrogenase

Matrix	Relative activity	
	Purified enzyme[a]	Whole cells[b]
Neutral acrylamide	100	100
Anionic acrylate/acrylamide	87	69
Cataionic MAPTAC/acrylamide	32	85

[a]Purified enzyme entrapped with a stabilizing concentration of BSA taken as 100. Specific activity of the preparation was 141 U/mg immobilized protein. All measurements were made at pH 5.5 (acetate buffer).

[b]Whole cells (glutaraldehyde-treated) entrapped by low-temperature radiation polymerization were taken as 100. Specific activity of the preparation was 0.71 U/mg protein in the bacterial cells. All measurements were made at pH 6.5, citrate–phosphate buffer.

long term stability of the enzyme and the immobilization coefficient but, more importantly, conserved our stocks of the enzyme. In comparison with the immobilized whole cells, all preparations showed higher specific activity (per mg immobilized protein).

pH vs activity profile. Immobilization of hydrogenase by absorbing it, along with a stabilizing concentration of BSA, onto polyacrylate–polyacrylamide beads resulted in a preparation with a pH profile (citrate–phosphate buffers) showing a maximum activity at pH 6.5 (Fig. 1). The free enzyme was maximally active at pH 6.0 under the same conditions. Whole cells on the other hand, were maximally active over the range pH 6.0–7.0. The pH maximum shift could be attributed to matrix charge effects.

Matrix charge effects. These effects on the immobilized enzyme were investigated by preparing low-temperature, radiation-polymerization entrapped samples and comparing them with similar samples prepared using whole cells. It was noted (Table 2) that both the purified enzyme and the whole cells were most active in neutral matrices. The purified enzyme lost about 13% of its activity when placed in an anionic matrix whereas it lost nearly 70% of its activity in a positively charged matrix. This effect was opposite to that found with the whole cell preparations.

Temperature stability. The temperature stability of the immobilized hydrogenase preparations was checked by determining the percent inactivation after high temperature incubation at pH 6.5. The results (Table 3) indicated that the purified enzyme lost 76% of its activity during the test period whereas all the immobilized preparations lost considerably less activity. The enzyme-BSA preparation entrapped in an acrylate/acrylamide matrix lost only 10% of its activity during the same period. A preparation containing the enzyme–BSA mixture immobilized by absorption lost much more activity (55%), suggesting that simple matrix charge effects were not sufficient to account for the retention of activity.

In comparison with the whole cell preparations, the purified enzyme was less stable. Immobilization of the whole cells further improved hydrogenase stability.

TABLE 3
Percent Inactivation of Hydrogenase Activity after Exposure to 65°C, 30 min, pH 6.5

Preparation	Inactivation[a] %
Purified enzyme	76
Immobilized enzyme	
Entrapped in acrylamide	21
acrylate/acrylamide	11
DMAPMA/acrylamide	45
Surface bound[b]	55
Whole cells	53
Immobilized whole cells	
Entrapped in acrylamide	17
acrylate/acrylamide	17
DMAPMA/acrylamide	35

[a]Activity measurements were all relative to the starting preparation. Enzyme preparations (10 μg) were incubated in 0.1% w/v BSA solutions (0.1*M* phosphate) and activities were measured at pH 5.5 (acetate). Whole cell preparations (1% w/v) were incubated in phsophate buffer (0.1*M*) and activities were measured at pH 6.5 (phosphate).

[b]Prepared by absorbing hydrogenase with a stabilizing amount of BSA onto low-temperature radiation-polymerized acrylate/acrylamide beads.

As had been noted with the purified enzyme, neutral and anionic matrices were more conducive to retention of activity.

Oxygen stability. In no case could solutions containing the chemically reduced (dithionite) enzyme have their activity revived after exposure to oxygen (*14*). Immobilized preparations of both purified enzyme and whole cells would be revived after exposure to oxygen. For example, immobilized enzyme preparations (entrapped and absorbed) retained approximately 10% of their initial activity after stirring in the presence of air for 5 min. Immobilized whole cells, on the other hand, retained approximately 25% of their initial activity under the same conditions.

Long-term storage. Hydrogenase activity remaining after storage of the various preparations in phosphate buffer at 4°C, in the presence of air, is given in Table 4. After 60 d storage, the purified enzyme and whole cells had lost 60–70% of their original activity, but the immobilized preparations had much better retention of activity. The best preparations were obtained when the purified enzyme was absorbed and crosslinked on the porous ion exchange beads or when whole cells were entrapped in an acrylate/acrylamide matrix. Simple entrapment of the en-

TABLE 4
Percent of Initial Hydrogenase Activity Remaining after Long-Term Storage[a]

Preparation	Initial activity remaining after 60 d, %
Enzyme	39
Immobilized enzyme	
Absorption	84
Entrapment	47
Whole cells	23
Immobilized whole cells	
Entrapment in acrylate/acrylamide	79
acrylamide	65
MAPTAC/acrylamide	56

[a]Preparations were stored at 4°C in sodium phospate buffer (0.1*M*, pH 6.5) for 60 d. Activities (μmol H_2/min/mg protein immobilized) were measured at pH 5.5 (acetate).

zyme or placing whole cells in a cationic environment led to larger losses of activity.

Conclusions

The results show that immobilization of the purified hydrogenase enzyme from *D. desulfuricans* has an effect on the pH profile and the thermal and oxygen stability of the enzyme. Matrix charge effects also affected the relative activity of enzyme preparations as well as the long-term storage stability of the enzyme. The preparations studied here may prove useful in the chemical and photochemical hydrogen utilization and generation systems described previously (*4–8*).

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