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Thermal Stability of a *Desulfovibrio gigas* Periplasmic Hydrogenase in Reverse Micelles

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ABSTRACT

The thermal and operational stabilities of *Desulfovibrio gigas* periplasmic hydrogenase were studied at 30 and 50°C in aqueous and micellar media. At the lower temperature the hydrogenase was more stable in reverse micelles of a cationic surfactant. No inactivation was detected over almost 16 d of incubation of the hydrogenase in the reverse micellar solution, during which the enzyme lost around 50% of the initial enzymatic activity in the aqueous solution. At 50°C the hydrogenase was more stable in aqueous medium, because of the changes that occur with the temperature in the organic phase—precipitation of surfactant and solvent evaporation. It was found that only micellar solutions of this enzyme can be repeatedly used, since the hydrogenase is inactivated after the first cycle of oxireduction in aqueous medium. The effect of glycerol and the electron carrier methylviologen as stabilizers of hydrogenase activity was also investigated. The results are interpreted on the grounds of hydrogenase and surfactant electrostatic properties.

Index Entries: Hydrogenase; reverse micelles; stability; oxireduction reactions; organic media.

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INTRODUCTION

The use of enzymes in organic solvents, in addition to the conventional aqueous medium, opened a new area of study in enzymatic catalysis, with promising applications in biotechnology (*1,2*). An important aspect of using microencapsulated enzymes is the study of enzyme stability in reverse micelles of surfactants in organic solvents. The retention of enzymatic activity upon encapsulation in reverse micelles is of upmost importance to the development of biotechnological processes that involve the use of biocatalysts in these novel solvent systems. However, few studies have been done in this area. A study of the stability of chymotrypsin in reverse micelles of tetradecyltrimethylammonium bromide (TTAB) in heptane/chloroform revealed that this enzyme was more stable at low W_o ([H_2O/[Surfactant]) (*3*). A lipase from *Chromobacterium viscosum* was more stable in reverse micelles of sodium dioctilsulfosuccinate (known as AOT) in isooctane than in aqueous medium (*4*). Hydrogenase from *Desulfovibrio vulgaris* has been used for the production of hydrogen and the regeneration of cofactors (*5*) in reverse micelles of hexadecyltrimethylammonium bromide (CTAB). The activity and stability of *Desulfovibrio gigas* hydrogenase in AOT reverse micelles were also studied (*6,7*). This enzyme displayed a low activity and a poor operational stability in AOT reverse micelles, when compared with results in aqueous medium.

Additives, such as enzyme ligands, neutral organic compounds, and ionic compounds, have been used to stabilize enzymes in aqueous media against high temperatures or other denaturing conditions (*8*). Glycerol has been one of the polyols more extensively used as a stabilizing agent for proteins. It can also be effective in organic solutions. The presence of 30% glycerol enhanced chymotrypsin stability fourfold in reverse micelles of TTAB in heptane/chloroform (*3*). This work reports data on the thermostability and operational stability of hydrogenase from *Desulfovibrio gigas* encapsulated in reverse micelles of an anionic surfactant (AOT) and a cationic surfactant (CTAB). We evaluated the effect of glycerol and the electron carrier viologen dye as stabilizers of the hydrogenase catalytic activity.

MATERIALS AND METHODS

Enzyme

Desulfovibrio gigas periplasmic hydrogenase (EC 1.12.2.1) was purified by the method described by (*9*). This enzyme has a mol wt of 89,000 kDa and a isoelectric point of 4.5.

Chemicals

The anionic surfactant sodium bis[2-ethylhexyl]sulfosuccinate (AOT) and the cationic surfactant hexadecyltrimethylammonium bromide (CTAB) were from Sigma (St. Louis, MO), and the organic solvents, analytical grade, from Merck (Darmstadt, Germany). Glycerol, 99% purity was from Sigma. Sodium dithionite 87% purity, and sodium azide 99% purity were from Merck. The residual O_2 concentration in the H_2 gas was less than 5 ppm.

Micellar Systems

Hydrogenase was encapsulated in reverse micelles by the injection method (*10*). A buffered aqueous solution of this enzyme (20 m*M* Tris-HCl, pH 7.6) was injected into a vortex stirred micellar solution of 0.2*M* CTAB in octane containing 11% (v/v) hexanol, 2% (v/v) water (20 m*M* Tris-HCl, pH 7.6 buffer), or 0.2*M* AOT in octane.

Activity Assays

The hydrogenase activity was measured spectrophotometrically at 600 nm using methylviologen as electron acceptor with $\epsilon = 10{,}000 M^{-1}$ cm^{-1} in the reduced form of the enzyme (*7*), according to the following scheme:

$$H_2 + 2\,MV^{2+} \longrightarrow 2\,H^+ + 2\,MV^+$$

The reaction was started by supplying H_2 to the enzyme and electron carrier solution.

Determination of Optimum W_o of Activity

For the determination of the optimum W_o (the molar ratio of water to surfactant concentrations in a reverse micellar system) of activity, the hydrogenase was first activated under an atmosphere of H_2 for 4 h. Different volumes of hydrogenase and methylviologen solutions were then encapsulated in CTAB reverse micelles (final concentration: [H_2ase] = 0.45 μM and [MV] = 25 m*M*), and the activity tested. The reaction started 2–5 min after the H_2 supply.

Thermal and Operational Stability Studies

The hydrogenase (1 μM) was encapsulated at the optimum W_o in a micellar solution of CTAB, W_o = 19.6, and AOT, W_o = 11.1 (*7*). These solutions were then incubated at the desired temperature (30 or 50°C) with or without 10% glycerol and/or 25 m*M* methylviologen, and the activity tested at several time intervals. These studies were also performed with the enzyme in aqueous solution ([H_2ase] = 0.044 μM; [MV] = 1 m*M*).

Sodium azide was added to the enzymatic assays in aqueous solution to prevent microbial contamination of the enzyme solution, when the hydrogenase was incubated separately from the viologen dye, during the several days of incubation. This procedure was not necessary when the effect of the substrate on the enzyme stability was studied, since methylviologen prevents also the microbial growth in aqueous hydrogenase solutions. Sodium azide did not interfere with the enzymatic assay.

For the operational stability studies, 1 mL of micellar solution of CTAB or AOT, ([H_2ase] = 15 μM; [MV] = 25 mM), or aqueous solution ([H_2ase] = 0.6 μM; [MV] = 1 mM) was incubated at the desired temperature, 30 or 50°C, and the activity tested every 2.5 h with the same solution, i.e., the enzyme was repeatedly used. Between two measurements of activity the hydrogenase was reoxidized in open air. The effect of glycerol (10%) and methylviologen (25 mM) was also tested. These additives had no effect on the initial activity of hydrogenase in either aqueous or organic media. A cell with agitation (Hellma CUV-O-STIR Model 333) was used for the activity assays in aqueous medium owing to the speed of the reaction. This was not necessary for the reactions measured in micellar media.

RESULTS AND DISCUSSION

Effect of W_o on the Hydrogenase Activity

The hydrogenase was easily encapsulated in reverse micelles of the anionic or cationic surfactants, despite its large mol wt (89,000 Da) and, in the case of the anionic surfactant AOT, of unfavorable electrostatic interactions at the pH studied (pI = 4.5 [*6*]), i.e., the charge of the surfactant and the protein is of the same type. It is likely that this protein creates its own microenvironment inside the aggregates, that is, the encapsulation of the hydrogenase may occur through the mobilization of several micelles and not only one. It is also possible that hydrophobic interactions occur between the alkyl chains of the surfactant and hydrophobic residues of the protein. The activity of enzymes encapsulated in reverse micelles depends, among other factors, on the amount of water in the micellar aqueous pool. The hydrogenase activity was tested in CTAB reverse micelles at different W_o (W_o = [H_2O]/[Surfactant]). The results were compared with the ones obtained in a previous work (*6*), and are shown together in Fig. 1. The profile of activity is sharper for the enzyme encapsulated in anionic micelles, which suggests that the hydrogenase is more sensitive to its microenvironment in AOT micelles. Also the maximum activity attained in the cationic micellar medium (0.7 μmol H_2/min·mg) was higher than in anionic micelles (0.2 μmol H_2/min·mg). However, the activity retention for the encapsulated enzyme was low for both surfactants, less than 2% of the value in aqueous solution (45 μmol H_2/min·mg), owing probably, to the residual oxygen concentration in the organic solvent.

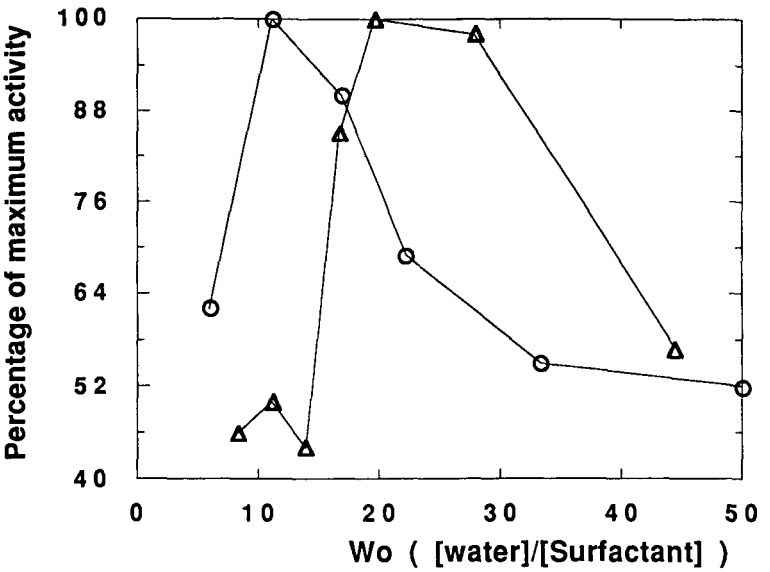

Fig. 1. Effect of the micellar water content (W_o = $[H_2O]$/[Surfactant]) in the hydrogenase activity at 30°C in anionic (○, AOT) (results from Castro and Cabral [1989a]) and cationic (△, CTAB) micellar media. The enzyme was activated by H_2 4 h before the measurement of the activities above. [H_2ase] = 0.45 μM; [MV] = 25 m*M* in the aqueous pool, at pH 7.6 (20 m*M* Tris-HCl).

This can be explained by the lower solubility of O_2 (an inhibitor of almost all hydrogenases) in water (80 times lower than in a hydrocarbon solvent.

Thermal Stability

The hydrogenase thermostability was studied at 30 and 50°C in aqueous and micellar solutions (Fig. 2A; Table 1). The effect of glycerol and methylviologen as stabilizers of the hydrogenase activity was also tested (Fig. 2B,C; Table 1). The results indicate that the enzyme is more stable in reverse micelles of CTAB than in aqueous solution, and was rapidly denatured in AOT reverse micelles (Fig. 2A). Several factors may contribute to this. The cationic micellar system is different from the anionic in three ways: type and charge of the surfactant; presence of a cosurfactant (hexanol); and higher volume of the aqueous phase at optimum W_o. At the pH studied the protein bears a global negative charge and therefore its encapsulation should be energetically more favorable in cationic micelles than in anionic ones. According to this, the enzyme was more stable and active in reverse micelles of CTAB (Fig. 2A). Thus the surfactant charge may determine the hydrogenase stability. It is known that acidic proteins, like the hydrogenases, are hydrophilic, which can explain the higher activity and stability in CTAB micelles, owing to the higher water content in these micelles at the optimum W_o. From Fig. 1 it can also

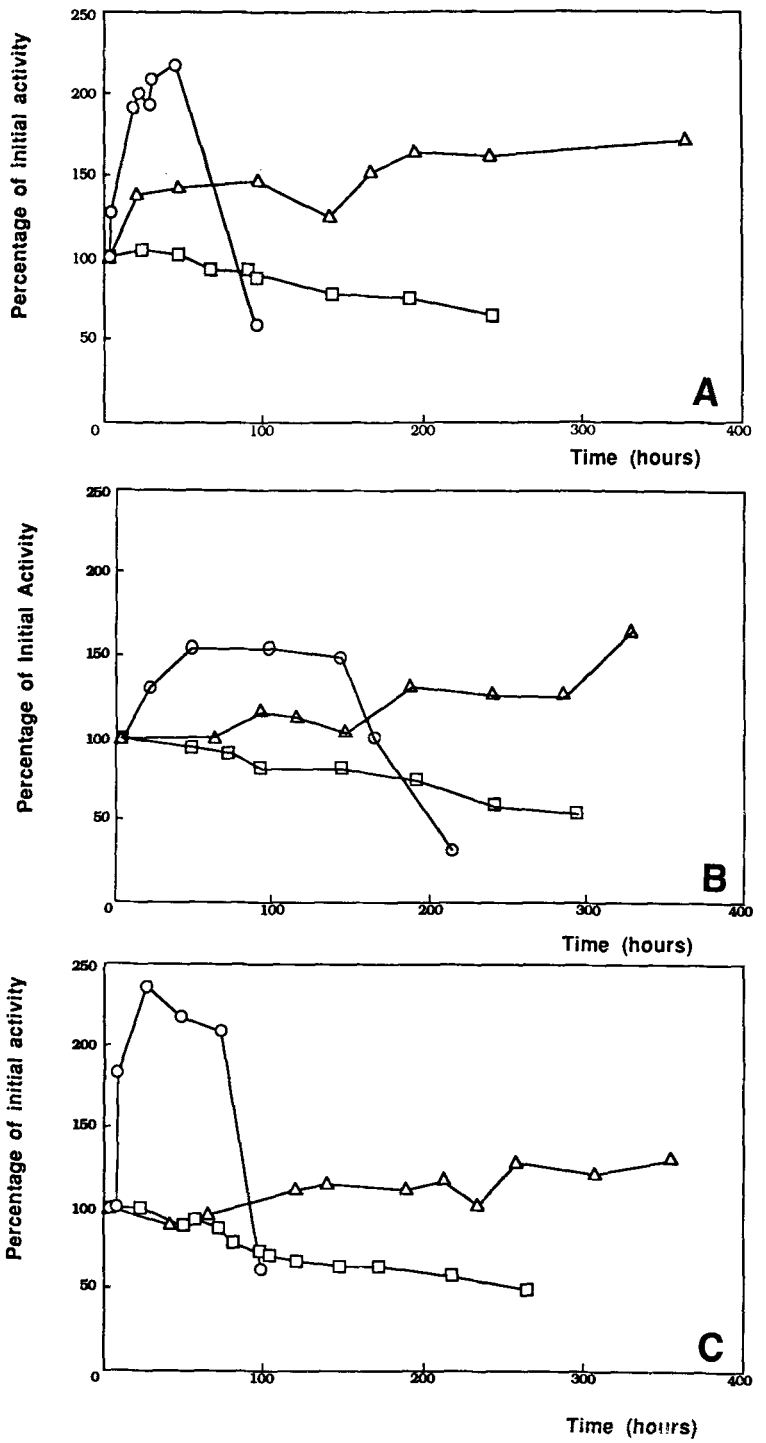

Fig. 2. (**A**) Thermostability of hydrogenase at 30°C in aqueous (□) and micellar media. The hydrogenase was encapsulated in anionic (○, AOT) and cationic (△, CTAB) micelles. [H_2ase] = 1 μM; [MV] = 25 mM in reverse micelles (pH 7.6, 20 mM Tris-HCl, in aqueous pool) and [H_2ase] = 0.044 μM; [MV] = 25 mM in aqueous medium (pH 7.6, 20 mM Tris-HCl). (**B**) Effect of glycerol (10% [v/v]) in the hydrogenase thermostability in aqueous medium (□) and micellar media (○, AOT; △, CTAB). (**C**) Effect of 25 mM methylviologen in the hydrogenase thermostability in aqueous medium (□) and micellar media (○, AOT; △ CTAB).

Table 1
Effect of Glycerol and Methylviologen
in the Thermostability of Hydrogenase at 50°C[a]

	Hydrogenase experimental half-life time, h			
Medium		Glycerol	MV	Glycerol + MV
Water	150	175	160	160
CTAB	55	70	65	100

[a]With 1 μM H_2ase and 25 mM MV in the aqueous pool of reverse micelles (pH 7.6, 20 mM Tris-HCl) or 0.044 μM H_2ase and 25 mM MV for the assays in aqueous medium (pH 7.6, 20 mM Tris-HCl).

Table 2
Initial Activities for the Free (in Aqueous Medium)
and Encapsulated Hydrogenase (in Reverse Micelles)[a]

	Initial enzymatic activity, μmol H_2/min·mg	
System	30°C	50°C
Water	45	115
AOT	0.20	0.40
CTAB	0.70	1.7

[a][H_2ase] = 0.044 μM; [MV] = 25 mM in aqueous medium (pH 7.6, 20 mM Tris-HCl), and [H_2ase] = 1 μM; [MV] = 25 mM in micellar media (pH 7.6, 20 mM Tris-HCl, in aqueous pool).

be seen that the hydrogenase is more sensitive to changes in its microenvironment when inside AOT micelles. The stability experiments were performed over a period of several days, during which changes occurred in the micelle structure owing to the slow evaporation of the solvent. That would cause shifts in the W_o of the solution incubated, and, implicitly, the destabilization of the protein. On the other hand, it is known that the anionic surfactant AOT is very aggressive to biological molecules. The spectroscopic properties of proteins are often changed on their encapsulation in reverse micelles of this surfactant (*10,11*). Table 2 indicates the initial activity of the hydrogenase in aqueous and reverse micellar solutions at 30°C. It can be seen that the enzyme is far more active in aqueous medium than in reverse micelles. However, it should be noted that these values may be underestimated. In all these experiments the hydrogenase was not activated, i.e., it was used in the oxidized state. In these conditions, for the enzyme encapsulated in reverse micelles, the reaction only starts approx after 1 h of incubation under H_2, whereas in aqueous solution that lag is reduced to 5 min. During this period of time there is solvent evaporation and agitation of the reaction medium, which can both denature the protein. Also, the oxygen inhibition of the hydrogenase is a more serious problem for the enzyme encapsulated in reverse

micelles than in aqueous solution because of the higher solubility of this gas in organic solvents. All these factors can contribute to a slow denaturation of the encapsulated protein during the incubation period under H_2, and to a measured activity lower than the real one.

The stability studies were also conducted at 50°C. At this higher temperature the hydrogenase was more stable in aqueous solution than in CTAB reverse micelles (Table 1). This can be interpreted as a destabilization of the micelle structure over time owing to solvent evaporation and/or surfactant precipitation, causing the protein exposure to the organic solvent and hence its denaturation. This was visible in some experiments where the octane and hexanol evaporation caused CTAB precipitation in the test tubes. The hydrogenase had a half life time lower than 15 min when encapsulated in AOT reverse micelles at this temperature (results not shown).

The increase in temperature also induces changes in the physicochemical properties of the micellar system that can perturb the conformation of the encapsulated hydrogenase and therefore alter its catalytic activity. At this temperature the collisions between micelles should increase, exposing the enzyme to the organic solvent more often, and therefore causing its denaturation. There seems to be evidence for the existence of short range attractive forces between reverse micelles, i.e., upon the collision of two aggregates the micelles stay together for a finite period of time. These forces increase in importance as the temperature is raised (until the upper temperature stability limit of the single phase reverse micellar system is approached) and are the result of interactions between the surfactant and the solvent (*11*). At higher temperatures, the time and number of contacts between micelles increase (*11*) and the surfactant can interact with the protein for a more extended period of time, causing its denaturation.

The effect of additives was also studied at 30 and 50°C. At 30°C, glycerol stabilized twofold the hydrogenase encapsulated in AOT reverse micelles (Fig. 2B). The substrate, methylviologen had little effect on the stabilization of this enzyme (Fig. 2C). At 50°C glycerol and methylviologen had a synergistic effect in the stabilization of hydrogenase encapsulated in CTAB reverse micelles (twofold stabilization) (*see* Table 1). It is known that the substrate stabilizes the enzyme by binding to the native state of the protein (*8*). Polyols like glycerol have a stabilizing effect by changing the structure of water in the vicinity of the protein molecule (*12*).

Operational Stability

The hydrogenase stability was also studied at 30 and 50°C in terms of consecutive oxidation reduction cycles, i.e., the operational stability. The enzyme was assayed every 2.5 h and only the micellar solutions could be used several times. The results are shown in Figs. 3 and 4. In micellar

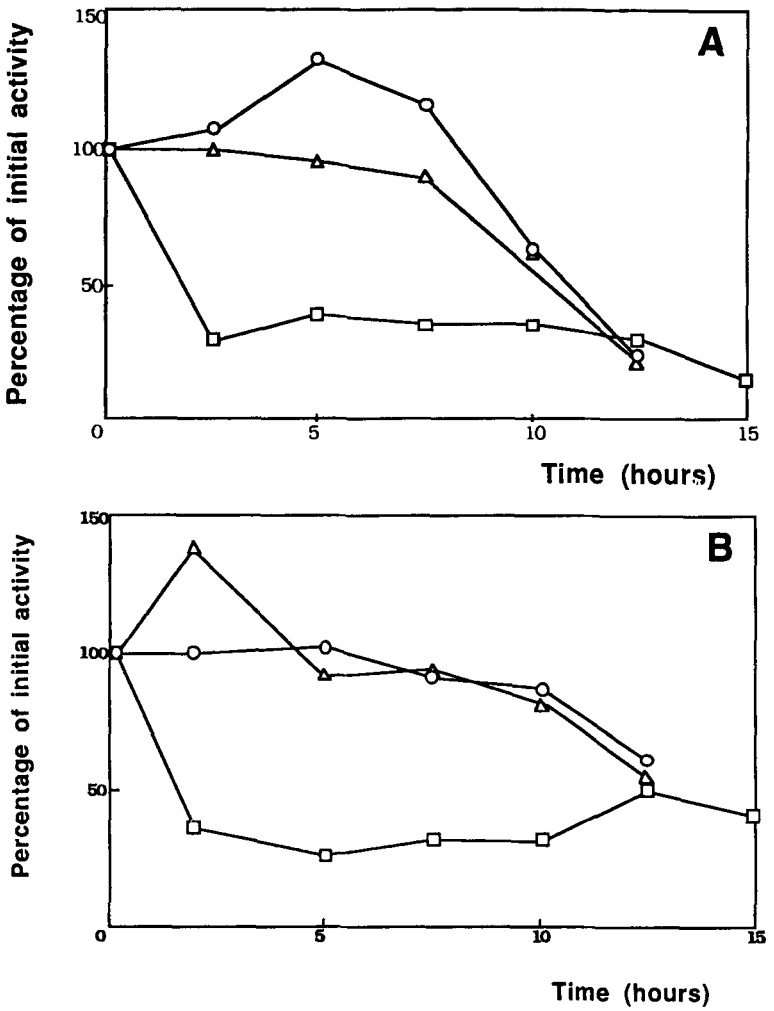

Fig. 3. (**A**) Operational stability of hydrogenase in aqueous (□) and reverse micellar media (○, AOT; △, CTAB) at 30°C. The same hydrogenase solution was used every 2.5 h. [H_2ase] = 15 μM; [MV] = 25 mM in reverse micelles (pH 7.6, 20 mM Tris-HCl, in aqueous pool), and [H_2ase] = 0.6 μM; [MV] = 1 mM in aqueous medium (pH 7.6, 20 mM Tris-HCl). (**B**) Effect of glycerol (10% [v/v]) in the operational stability in aqueous (□) and micellar media (○, AOT; △, CTAB).

media the hydrogenase is still active after five of these cycles before losing the same percentage of activity as the enzyme in aqueous medium after the first cycle (Fig. 3A). Glycerol enhanced the hydrogenase stability by one more cycle in the micellar solutions (Fig. 3B).

Similar results were obtained at 50°C (Fig. 4), but the hydrogenase activity decayed more rapidly as expected, because of the higher temperature and/or the consequent higher evaporation of the organic solvent and destabilization of the micelle structure. In aqueous medium the hydrogenase loses more than 50% of the initial activity during the first

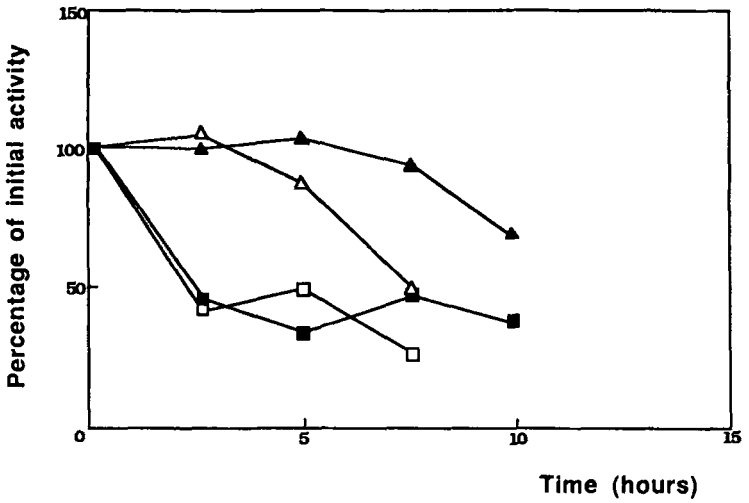

Fig. 4. Operational stability of hydrogenase in aqueous (□) and cationic reverse micellar medium (△) at 50°C. The effect of glycerol (10% [v/v]) was also studied (▲, ■). [H_2ase] = 15 μM; [MV] = 25 mM in reverse micelles (pH 7.6, 20 mM Tris-HCl, in aqueous pool) and [H_2ase] = 0.6 μM; [MV] = 1 mM in aqueous medium (pH 7.6, 20 mM Tris-HCl).

cycle, but from that time on, the enzyme remains stable, probably owing to a self stabilization by the denatured protein (*13*). This effect should be more important in these experiments than in the ones of thermal stability, because of the higher protein concentration used (15-fold higher).

CONCLUSIONS

The hydrogenase exhibited a very low activity in the micellar solutions, as compared with the activity values obtained in aqueous solution, but its stability improved when used in a cationic micellar system. This enhanced stability was not observed at 50°C because of alterations in the structure of the micellar phase. Of great interest was the fact that only micellar solutions of hydrogenase could be used several times, whereas the enzyme in aqueous solution was inactivated after one cycle of oxi-reduction of methylviologen. The increase in thermostability of hydrogenase observed in the cationic micellar system, as opposed to the fast inactivation of hydrogenase activity in the anionic micellar system, was interpreted as a consequence of favorable electrostatic interactions of the hydrogenase with the polar heads of the cationic surfactant. Thus, the surfactant charge may determine the stability of an enzyme in a micellar solution, although other factors may be involved, such as molecular toxicity of the surfactant and organic phase, and phase toxicity owing to the agitation of the organic solution during the measurement of enzymatic activity.

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