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ORIGINAL PAPER

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Increased stability of malate dehydrogenase from *Halobacterium salinarum* at low salt concentration in reverse micelles

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Abstract The stability of malate dehydrogenase (hMDH) from *Halobacterium salinarum* in aqueous medium at low salt concentrations (1 and 0.5 M NaCl) was studied at 4° and 25°C. The results showed that hMDH was more stable at the higher salt concentration and the low temperature. hMDH was introduced into reverse micelles of hexadecyltrimethylammonium bromide in cyclohexane with 1-butanol as cosurfactant. The hMDH stability in this system was studied at two ω_0 ([H_2O]/[surfactant]) values and the effects of salt concentration, presence of substrate and dilution before or after its introduction into reverse micelles were examined. The results showed that the half-life of hMDH dissolved in buffer with 1 M NaCl was 12–50 days in reverse micelles (depending on the various conditions), in contrast to only about 1 day in aqueous medium at 25°C. These observations indicate that reverse micelles provide a microenvironment that allows a much greater stability of this enzyme compared with an aqueous medium.

Key words hMDH · Halophilic enzyme · CTAB · Reverse micelles · Stability

Introduction

Extreme halophiles (Halobacterium) require a high salt concentration for optimal growth (Lanyi 1974). Their intracellular milieu contains nearly saturated KCl and high NaCl concentrations. Thus, it is understandable that their enzymes are halophilic, i.e., they depend on salt in the molar range for their activity and stability. The stability of halophilic proteins in aqueous buffer solutions has been studied intensively (Pundak et al. 1981; Hecht and Jaenicke 1989; Bonet et al. 1991; Bonnete et al. 1994; Dym et al. 1995; Elcock and McCammon 1998; Mevarech et al. 2000). Of the halophilic enzymes studied, the best characterized is malate dehydrogenase (hMDH) (Dym et al. 1995). The thermal stability of hMDH has been investigated in a variety of solvent conditions (Bonnete et al. 1994; Ebel et al. 1999) and as a function of both salt concentration and pH (Hecht and Jaenicke 1989; Madern and Zaccai 1997). The results of these studies have shown that the stability of hMDH increases with increasing salt concentration and with a decrease in temperature.

Reverse micellar systems are produced by the use of a surfactant in an organic medium. The micelles contain very small amounts of water with peculiar properties, many of which mimic those of water in contact with biological membranes (Luisi et al. 1988). The amount of water in each micelle and, hence, its size is determined by ω_0, a parameter defined as the water/surfactant molar ratio. In the past decade much experimental evidence has demonstrated that a variety of enzymes can perform their catalytic activity within reverse micelles, sometimes exhibiting certain peculiar features such as superactivity and change of specificity (Ruckenstein and Karpe 1990; Verhaert et al. 1990; Fadnavis et al. 1993; Setti et al. 1995).

Although some enzymes display a similar stability in both aqueous and reverse micellar media (Walde et al. 1988; Peng and Luisi 1990), most enzymes increase their stability and retain their catalytic activity for more prolonged periods when introduced into reverse micelles (Fadnavis et al. 1993; Martinek et al. 1981; Hilhorst et al. 1982; Larsson et al. 1987; Hayes and Gulari 1994; Khmelnitski et al. 1992; Crooks et al. 1995). However, other enzymes are inactivated in reverse micellar medium (Fadnavis et al. 1993; Han and Ree 1987; Khmelnitski et al. 1992).

In a previous study, hMDH from *Halobacterium salinarum* was introduced into reverse micelles of hexade-

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cyltrimethylammonium bromide (CTAB) and its behavior was characterized (Piera-Velazquez et al. 2001). Here, we compared the stability of this malate dehydrogenase in aqueous medium and in reverse micelles and evaluated the effects of varying salt concentrations and the size of the micelles. We also examined the effects of the presence of substrate or coenzyme in the micellar system on its activity and stability. We observed that this halophilic enzyme can maintain its stability in reverse micelles for extended periods of time at very low salt concentrations. The increased stability in reverse micelles we demonstrated here allows the study of halophilic enzyme kinetic behavior under conditions in which essentially no activity can be detected in aqueous solution. Furthermore, the extremely long half-life of enzyme activity in reverse micelles that we observed should widen the practical applications of hMDH and other catalysts which display similar activity stabilization when introduced into reverse micelles.

Materials and methods

Chemicals

CTAB, approximately 99% pure, was obtained from Fluka (Buchs, Switzerland). NADH (grade II) was from Boehringer Mannheim (Mannheim, Germany). Oxalacetic acid (OAA) and organic solvents were from Sigma (St Louis, MO, USA) and were used without further purification. All salts employed were of analytical grade. Cations were used as chlorides.

Enzyme preparation

A colorless mutant of *H. salinarum* (NRC 36014) was used. Cultures were grown at 37°C in 5 l batches of 0.5% yeast extract in a 25% mixture of salts with continuous stirring and aeration as previously described (Rodriguez-Valera et al. 1980). The cells were harvested in the late exponential phase by centrifugation at 5,000 *g* for 30 min, the pellet was resuspended in 50 mM sodium phosphate buffer, pH 6.6, containing 2.5 M ammonium sulfate, and sonicated in an ultrasonic disruptor. The disruptor suspension was centrifuged at 105,000 g for 60 min at 4°C. The supernatant was used as a crude enzyme preparation. Subsequent purification of the enzyme was performed with a modification of the method described by Mevarech et al. (1977). Briefly, three steps were followed: (1) Sepharose–ammonium sulfate chromatography on a 5×50 cm Sepharose 4B column (Pharmacia, Sweden) equilibrated with 50 mM sodium phosphate buffer, pH 6.6, containing 2.5 M ammonium sulfate and eluted with a descending linear gradient of ammonium sulfate from 2.5 to 0.5 M; (2) gel-permeation chromatography on a 2.7×26 cm Sephadex G100 column equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.3, containing 2 M NaCl; and (3) adsorption and dilution in hydroxyapatite which was equilibrated with 50 mM sodium phosphate buffer, pH 7.3, containing 4.26 M NaCl and diluted at different concentrations of sodium phosphate from 0.05 to 0.3 M. Although the final preparation was not homogeneous by electrophoretic criteria, only minor contaminants were present. The purified enzyme solution was brought up to either 1 or 0.5 M NaCl concentration by dialysis prior to measurements of enzymatic activity or introduction into micelles. The concentration of purified enzyme utilized in all experiments was 0.01 mg/ml.

Enzymatic activity in aqueous buffer

The standard assay mixture consisted of 0.1 ml of enzyme solution and 2.5 mM OAA (final concentration) in 50 mM sodium phosphate buffer, pH 7.3, containing 4.26 M NaCl, 5 mM EDTA and 1 mM 2-mercaptoethanol. The solution was incubated at 35°C. The reaction was initiated by adding 0.05 ml of coenzyme solution (final concentration 0.25 mM) and NADH oxidation was monitored at 340 nm. An absorption coefficient of 5,370 M^{-1} cm^{-1} was used to determine NADH concentration.

Preparation of microemulsions

The surfactant/solvent solution consisted of 0.2 M CTAB in cyclohexane containing 1-butanol (1 M) as cosurfactant. The microemulsion was prepared by adding the various constituents under constant stirring at 25°C. The buffer component was 50 mM sodium phosphate buffer, pH 7.3, containing either 1 or 0.5 M NaCl and 5 mM EDTA and 1 mM 2-mercaptoethanol.

Preparation of reverse micelles

Two micellar solutions were prepared, one containing enzyme solution (36 µl/ml microemulsion for ω_0 10 and 54 µl/ml microemulsion for ω_0 15) and the other containing the same volumes of a solution of enzyme and OAA (1 mM). These solutions were incubated at 25°C. A third system was prepared containing 1 mM OAA and NADH (5 mM). The three systems were prepared so that they had the same $\omega_0([H_2O]/[surfactant])$. The solutions were then vigorously mixed until they became a clear single phase by visual inspection. All concentrations are given with respect to the total aqueous volume because the reaction occurs in this phase.

Enzyme activity measurements

Reactions were initiated by mixing 1 ml of one of the two micellar solutions containing enzyme (or enzyme and substrate) and 2 ml of micelles containing OAA and NADH. Initial reaction rates were determined by monitoring the absorbance changes at 340 nm. An absorption coefficient of 4,560.5 M^{-1} cm^{-1} was used to determine NADH concentration in micellar solutions. All measures were carried out at 35°C.

Stability assays

The effect of salt concentration on the stability of hMDH was determined in both systems (aqueous medium and reverse micelles) by maintaining the enzyme in 0.05 M phosphate buffer, pH 7.3 at either 1 or 0.5 M NaCl concentration and constant temperature (either 5° or 25°C in aqueous medium and 25°C in micellar system). Aliquots of 1 ml were taken at different times and the residual enzymatic activity was assayed as described above.

The half-life time of enzyme activity in aqueous medium was calculated from the single exponential decay rate constant employing the GraFit program (Erithacus Software, version 3.0). We used the first and the last measured data to calculate the half-life of the enzyme in reverse micellar media.

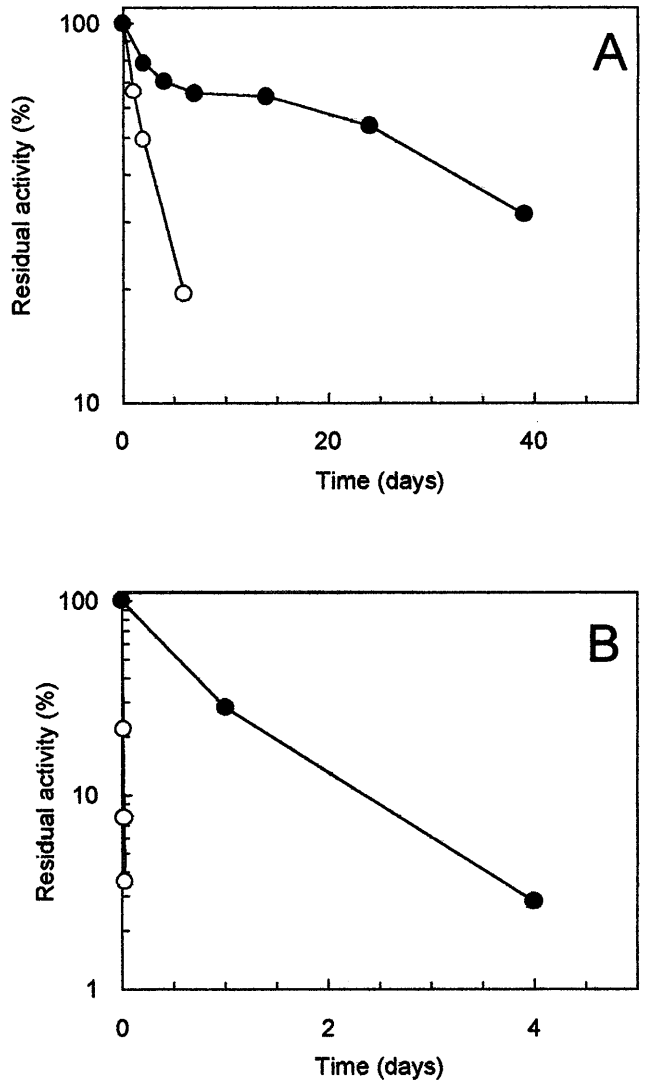

Fig. 1A,B. Stability of hMDH from *Halobacterium salinarum* in 50 mM sodium phosphate buffer, pH 7.3, containing 0.5 or 1 M NaCl, 5 mM EDTA and 1 mM 2-mercaptoethanol. Temperatures of incubation 5° (**A**) and 25°C (**B**). Salt concentrations: *open circles*, 0.5 M NaCl; *solid circles*, 1 M NaCl

Results

Enzyme stability in aqueous medium

The effects of temperature and salt concentration on the hMDH residual activity were measured after dialysis of the enzyme against phosphate buffer containing either 1 or 0.5 M NaCl, and incubation at either 5° or 25°C. Figure 1A shows the residual activity of the enzyme incubated at 5°C, and Fig. 1B the same assay performed at 25°C. The calculated values of half-life times obtained are shown in Table 1. We found that the activity of the enzyme decreased faster when the salt concentration of the medium was 0.5 M NaCl than when the salt concentration was 1 M NaCl at either temperature. The half-life time of enzyme activity in 1 M NaCl was approximately 30 times lower at 25°C than that at 5°C. At 25°C, the residual activity in 1 M NaCl was 350 times higher than at 0.5 M NaCl.

Enzyme stability in reverse micelles at high and low salt concentrations

The hMDH stability was studied in reverse micellar system at two ω_0 values (10 and 15) at 25°C. The effect of salt concentration on the stabilization of the enzyme in reverse micelles was also examined. Figs. 2 and 3 illustrate the residual activity of the enzyme dissolved in phosphate buffer containing 1 M NaCl and entrapped in reverse micelles prepared with solutions of either enzyme alone or enzyme plus OAA. The values presented in Table 2 show the half-life calculated from the data illustrated in Figs. 1, 2 and 3. The data showed a similar half-life at both ω_0 values when the enzyme was incubated with substrate, but the half-life was more than 10-fold that obtained in aqueous buffer. However, when the enzyme was incubated alone, the half-life was 4-fold greater at ω_0 15 than at ω_0 10.

The enzyme stability in the reverse micellar system when the enzyme was in buffer at the lower salt concentration (0.5 M NaCl) at the two ω_0 values (10 and 15) is presented in Fig. 4. In these experiments the enzyme was incubated in the presence of the substrate. The results show that the activity of the enzyme recovered after 13 days was approximately 35% at ω_0 10. In contrast, at ω_0 15 the activity decayed to less than 10% of the initial activity at the second day of incubation.

Table 1. Half-life ($t_{1/2}$ in days) of hMDH in aqueous buffer as a function of temperature and NaCl concentration

Temperature (°C)	Salt concentration	
	1 M NaCl	0.5 M NaCl
5	45.67 ± 6.00	1.47 ± 0.14
25	1.16 ± 0.12	0.0033 ± 0.0002

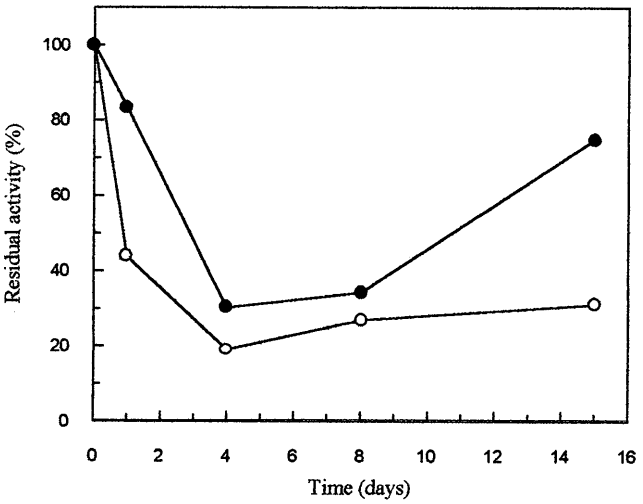

Fig. 2. Stability of hMDH from *H. salinarum* in CTAB/1-butanol/cyclohexane reverse micelles at two molar ratios of water to surfactant (ω_0). Micelles prepared with enzyme alone and dissolved in phosphate buffer containing 1 M NaCl: *open circles*, ω_0 10; *solid circles*, ω_0 15

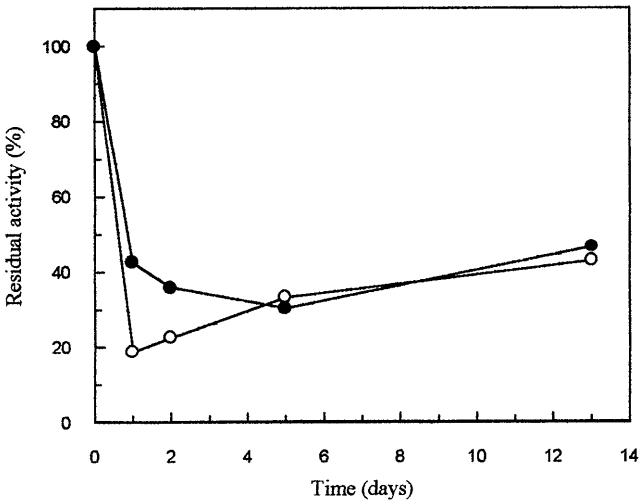

Fig. 3. Stability of hMDH from *H. salinarum* in CTAB/1-butanol/cyclohexane reverse micelles at different molar ratios of water to surfactant (ω_0). Micelles prepared with enzyme plus substrate and dissolved in phosphate buffer containing 1 M NaCl: *open circles*, ω_0 10; *solid circles*, ω_0 15

We then studied the influence of the presence of substrate or coenzyme in the reverse micellar system (with the components of the reaction dissolved in buffer with 0.5 M NaCl) on enzyme stability. These results are illustrated in Fig. 5. The half-lives calculated from the exponential decay rates are shown in Table 3. The lowest stability was found when the enzyme was incubated alone, indicating that the simultaneous presence of either substrate or coenzyme resulted in increased enzyme stability.

We also examined two different procedures for diluting the salt concentration from 1 to 0.5 M NaCl on the residual enzymatic activity in reverse micelles, and compared the

Table 2. Half-life ($t_{1/2}$ in days) of hMDH in aqueous buffer and reverse micelles (RM) with either enzyme alone (E) or enzyme and substrate (E-OAA) in the same micellar system, at ω_0 10 and ω_0 15, 25°C and 1 M NaCl

	$t_{1/2}$ (days)	
	ω_0 10	ω_0 15
RM (E)	12.82	51.72
RM (E-OAA)	16.47	18.18
Aqueous buffer	1.16	

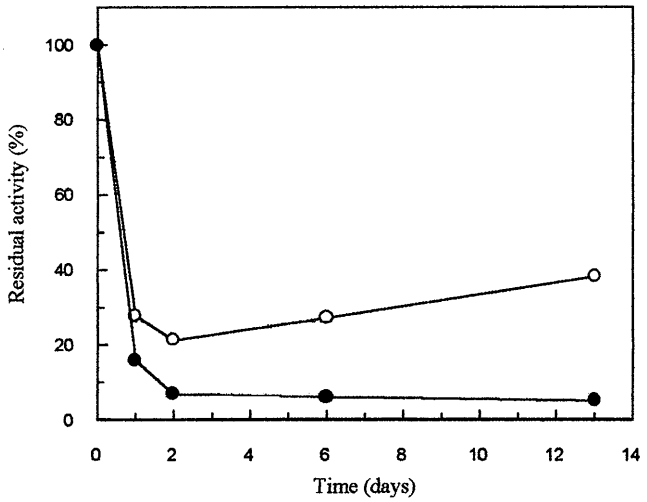

Fig. 4. Stability of hMDH from *H. salinarum* in CTAB/1-butanol/cyclohexane reverse micelles at different molar ratios of water to surfactant (ω_0). Micelles prepared with enzyme plus substrate and dissolved in phosphate buffer containing 0.5 M NaCl: *open circle*, ω_0 10; *solid circle*, ω_0 15

results to the stability of the enzyme in aqueous medium. In the first procedure the salt concentration was diluted before the enzyme was introduced into the micelles. In the second procedure, micelles containing enzyme at high salt concentration (1 M) were mixed with micelles containing buffer without salt. The results shown in Fig. 6 demonstrate that dilution before and during their introduction in the micelles resulted in retention of more than 10% of initial enzyme activity within 20 h. In contrast, hMDH in aqueous medium lost all its activity in less than 2 h. The calculated half-life times of the enzyme show that the half-time was from 27- to 35-fold greater in reverse micelles than in aqueous medium (Table 4).

Discussion

It is well accepted that when enzymes are isolated from their cell environment they become unstable and rapidly lose their activity. Within cells, the stability of an enzyme

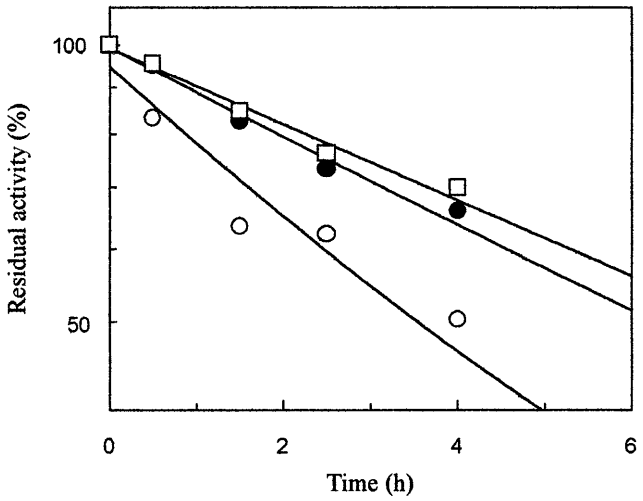

Fig. 5. Stability of hMDH from *H. salinarum* in CTAB/1-butanol/cyclohexane: *open circles*, reverse micelles prepared with enzyme alone; *solid circles*, enzyme plus substrate (OAA); *open squares*, enzyme plus coenzyme (NADH). Micelles prepared with phosphate buffer containing 0.5 M NaCl

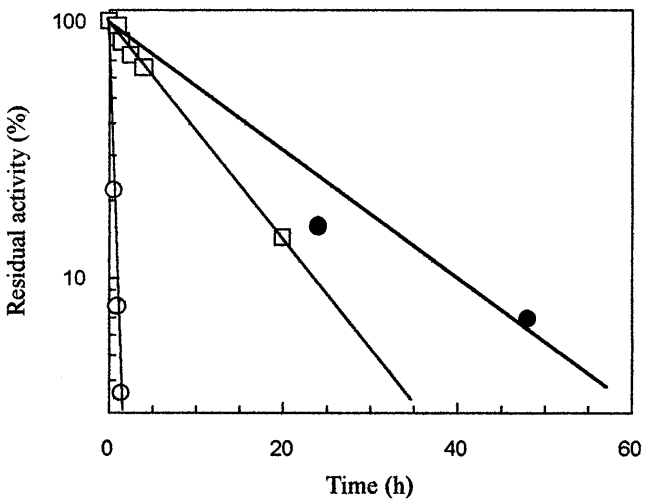

Fig. 6. Effect of the procedure of dilution to 0.5 M NaCl on the stability of hMDH from *H. salinarum* in CTAB/1-butanol/cyclohexane reverse micelles and in aqueous medium: *open circles*, aqueous medium; *solid circles*, dilution before the introduction of enzyme into micelles; *open squares*, micelles containing enzyme dissolved in 1 M NaCl mixed with micelles prepared with buffer containing no salt

Table 3. Half-life ($t_{1/2}$ in h) of hMDH in reverse micelles with enzyme alone (E) or enzyme and substrate (E-OAA) or coenzyme (E-NADH) in the same micellar system, at ω_0 15, 25°C and 0.5 M NaCl

	$t_{1/2}$ (h)
E	3.01 ± 0.53
E-OAA	5.78 ± 0.45
E-NADH	7.15 ± 0.85

Table 4. Effect of dilution of solution from 1.0 to 0.5 M NaCl before or within the micelles, ω_0 15 and 25°C on the half-life ($t_{1/2}$ in h) of hMDH in aqueous buffer and reverse micelles (RM)

	$t_{1/2}$ (h)
Aqueous buffer	0.21 ± 0.01
RM dilution before	7.37 ± 0.07
RM dilution within	5.78 ± 1.16

results from multiple interactions with cell components, and structured water in the cytoplasmic gel also plays an important role in its stability. In reverse micelles the water structure in the aqueous core provides a microenvironment in which multilayers of structured water exist (Luisi et al. 1988; Subramani et al. 1995; Tuena de Gomez-Puyou and Gomez-Puyou 1998). Zaccai and Cendrin (1989) proposed that the mechanisms of stabilization of halophilic MDH would be different depending on its environment; thus, reverse micelles offer a new medium for studying the behavior of this enzyme. The inactivation of hMDH at low salt is concomitant with dissociation and unfolding, but it is likely that in reverse micelles the enzyme maintains its conformation, even at low NaCl concentration.

The results of the studies described here show that stability of hMDH in aqueous solution is highly dependent on temperature ($t_{1/2}$ is about 40 times shorter at 25° than at 5°C in 1 M NaCl), but it is also markedly influenced by salt concentration ($t_{1/2}$ is about 30 times shorter at 0.5 than at 1.0 M NaCl at 5°C) as shown in Fig. 1 and Table 1.

The experiments carried out with hMDH maintained in reverse micelles in 1 M NaCl at 25°C for short periods of time showed that in both systems (micelles containing enzyme alone or enzyme with substrate) activity loss and denaturation are coupled processes showing the same trend with time (Figs. 2 and 3). However, when the enzyme is dissolved in buffer with 0.5 M NaCl, the activity was restored to 40% of initial activity at ω_0 10 value, whereas at ω_0 15 its activity was not restored (Fig. 4). We also found that enzyme stability increased with the amount of water within the micelles when the salt concentration was maintained constant (at 1 M NaCl), as shown in Fig. 2. In this experiment, increased stability was observed when the micelles are larger and there is more space inside them to accommodate a greater number of water molecules. These conditions allow greater binding of enzyme with water and salt molecules; therefore, increasing the stability of its conformation. However, at lower salt concentration (0.5 M NaCl) we found that enzyme stability decreases with ω_0. These results confirm the previous findings that the protein is stabilized by interactions with hydrated ions, i.e., the competitivity between salt ions and free water (Ebel et al. 1999).

When we examined the effect of substrate or coenzyme presence on stabilization of hMDH in reverse micelles we found that their presence in the reaction stabilized the enzyme (Fig. 5), possibly by induction of conformational changes caused by enzyme-coenzyme-substrate complex formation. It is known that the denaturing effect of surfac-

tants on enzymes can be substantially reduced by the addition of substrates (Khmelnitski et al. 1993).

When the enzyme was diluted from 1.0 to 0.5 M NaCl within the micelles, $t_{1/2}$ was about 25 times longer than in aqueous solution. This result showed a remarkable increase in stability. If the enzyme was diluted from 1.0 to 0.5 M NaCl prior to its introduction into the micelles, a marked recovery of its activity was observed following an initial reduction in activity. Regardless how the dilution to 0.5 M NaCl was carried out, the enzyme remained highly active for extended periods of time, i.e., several days, at 25°C. Experiments performed at different ω_0 values seemed to indicate that this parameter affects the rate of reactivation but not the stabilization brought about by the micellar medium.

The results presented in this report demonstrate that reverse micelles provide a favorable microenvironment to allow hMDH to retain its native conformation. The remarkable increase in enzyme stability within reverse micelles is very likely due to the fact that reverse micelles confer on the enzyme a rigid conformation owing to the highly structured position of water molecules within the micelles.

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