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The dependence of a halophilic malate dehydrogenase on ω_o and surfactant concentration in reverse micelles

Sonsoles Piera-Velázquez*, Frutos Marhuenda-Egea, Eduardo Cadenas

División de Bioquímica, Facultad de Ciencias, Universidad de Alicante, Apartado 99, 03080 Alicante, Spain

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Abstract

The halophilic malate dehydrogenase (hMDH) activity of *Halobacterium salinarum* was studied as a function of micelle size (ω_o), in cethyltrimethylammonium bromide (CTAB)/cyclohexane reverse micelles, with 1-butanol as cosurfactant. The velocity dependence of the ω_o profile depends on the buffer used, the surfactant concentration, and the salt concentration. In phosphate buffer, the activity increases with increasing water content, while in Tris/HCl buffer a bell-shaped profile is generally observed. Despite a slight change in the ω_o-activity profile, the enzymatic activity was higher at low salt concentration even when we employed a different buffer.

The ω_o value for the maximal activity (optimum ω_o) varies directly with the enzyme concentration. The hMDH activity in reverse micelles depends on the surfactant concentration and the dependence of the activity of this enzyme on the surfactant concentration, at constant ω_o, is different for each ω_o value.

Keywords: HMDH; Halophilic enzyme; Reverse micelles; CTAB; ω_o

1. Introduction

One of the most striking features in reverse micellar enzymology is the fact that the enzymatic activities vary depending on the system's water content, which is usually expressed in terms of ω_o ($(H_2O)/(surfactant)$). This factor controls the diameter of the water pool surrounding the enzymes. The numerous enzymes studied previously appear to display a very similar dependence. This dependence can present in three different forms: (a) the saturation curve in which the ω_o value does not have an effect on enzyme activity; (b) the bell shaped curve and (c) a curve in which enzyme activity decreases continuously as ω_o increases, due to decreases in the conformational mobility of the enzyme at low ω_o.

Almost all enzymes have been found to exhibit a bell-shaped dependence of the activity on ω_o. In general, the maximum activity is found to occur around that ω_o value at which the size of the reverse micelle is somewhat larger than that of the entrapped enzyme. This characteristic is commonly attributed to conformational changes of the solubilized enzymes [1], the state of the solubilized water, and the ionic nature of the surfactant head groups [2].

The rates of reactions catalyzed by enzymes solubilized in reverse micelles depend upon both the water and surfactant concentrations and not upon the degree of hydration or micellar diameter. However, it is possible that the reaction rate in a specific reverse micelle may be dependent on its diameter [3]. Buffer

* Corresponding author. Present address: Department of Medicine, Division of Rheumatology, Thomas Jefferson University, Philadelphia, PA 19107, USA. Tel.: +1-215-503-5565.
E-mail address: sonsoles.piera@mail.tju.edu (S. Piera-Velázquez).

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components have also been shown to exert a considerable effect on enzyme activity and ω_o-activity profile of lipases in reverse micelles [4].

Han et al., showed that the minimal ω_o-value for obtaining maximal activity depends on the enzyme concentration: the higher the enzyme concentration, the higher $\omega_{o,\,max}$ [5]. Similar results were observed in another study [6]. Also, it was shown that the activity of lactate dehydrogenase increases with increasing water content, reaching the highest value at $\omega_o = 60$ (solubilization limit) [7]. A study with DNA polymerase also showed the same tendency [8]. Our group had studied the dependency of the maximum reaction rate (V_{max}) of alkaline *p*-nitrophenylphosphate phosphatase from *Halobacterium salinarum* on the water content value ω_o, which showed a bell-shaped curve [9].

The concentration of surfactant, at a constant ω_o, is a parameter that determines the concentration of micelles in the system [10]. Since increasing the surfactant concentration at constant ω_o has no effect on the size of the water cavity of the micelle, the consequence of this change is a proportionate increase in interfacial area. The activity of water-soluble enzymes, such as α-chymotrypsin and trypsin [10], and alkaline phosphatase [11], typically has been shown to be independent of surfactant concentration, in contrast to the activity of membrane-bound enzymes which depends greatly on surfactant concentration.

In the present studies, we initiated the characterization of malate dehydrogenase, an halophilic enzyme, which plays an important role in the metabolism of halophilic bacteria, in a reverse micellar system, under conditions not studied previously. The aim of this study was to examine the behavior of this enzyme in this micellar system and to determine the influence of the characteristic parameters of this system on enzymatic activity.

2. Materials and methods

2.1. Chemicals

Cetyltrimethylammonium bromide (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, USA), and were used without further purification. All salts employed were of analytical grade. Cations were used as chlorides.

2.2. 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 [12]. The cells were harvested in the late exponential phase by centrifugation at 5000 g for 30 min, the pellet was resuspended in 50 mM sodium phosphate buffer, pH 6.6, containing 2.5 M ammonium sulphate, and sonicated in an ultrasonic disintegrator. The disrupter 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 [13]. Briefly, three steps were used: (a) Sepharose-ammonium sulphate chromatography on a 5 cm × 50 cm Sepharose 4B column (Pharmacia, Sweden) equilibrated with 50 mM sodium phosphate buffer, pH 6.6, containing 2.5 M ammonium sulphate and eluted with a descending linear gradient of ammonium sulphate from 2.5 to 0.5 M; (b) a gel-permeation chromatography on a 2.7 cm × 26 cm Sephadex G100 column equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.3, containing 2 M NaCl and (c) 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 at pH 7.3 containing 4.26 M NaCl.

The final preparation was not homogeneous by electrophoretic criteria, but only minor contaminants were present.

2.3. 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 either 50 mM sodium phosphate or 100 mM Tris/HCl buffer, pH

7.3, containing 1 M NaCl, 5 mM EDTA and 1 mM 2-mercaptoethanol.

2.4. Preparation of reverse micelles solution

Enzyme, coenzyme and substrate were dissolved in either phosphate or Tris/HCl buffer. To achieve a particular ω_o, varying amounts of enzyme, substrate, or coenzyme solution were added to a predetermined volume of the microemulsion (depending on the assay). Two micellar solutions were prepared, one containing enzyme plus OAA (1 mM) and the second containing NADH (5 or 2 mM, depending on the assay). The two solutions were prepared with the same ω_o. The solutions were then vigorously mixed until they became a clear single phase by visual inspection. Enzyme concentration was expressed in milligram per assay. All concentrations are given with respect to the total aqueous volume, because the reaction occurs in this phase.

To examine the influence of pH on ω_o profile, we used phosphate buffer at either pH 7.3 or 8, and Tris/HCl buffer at pH 9. The three buffers contained 1 M NaCl, 5 mM EDTA and 1 mM 2-mercaptoethanol, and the enzyme concentration was 0.015 mg per assay.

2.5. Enzyme activity measurements

Reactions were started by mixing 2 ml of the micellar solution containing enzyme and substrate and 1 ml of micelles of NADH. Initial reaction rates were determined by monitoring the absorbance changes at 340 nm. An absorption coefficient of 4560.5 M^{-1} cm^{-1} was used to determine NADH concentration in micellar solutions. All experiments were carried out at 35°C.

The values of V_{max} and K_m^{OAA} were determined by non-linear regression analyses of the corresponding Michaelis-Menten curves, using the GraFit computer program (Erithacus Software, version 3.0, Unicam Iberica SA, Spain).

3. Results

3.1. Influence of pH on ω_o-activity profile

The effects of pH on ω_o-activity profile are illustrated in Fig. 1. The ω_o-activity profile with micelles prepared in buffers at three different pH: 7.3 and 8 in phosphate buffer and pH 9 in Tris/HCl buffer is shown. The activity of enzyme at a given ω_o increased with increasing water content until it reached the solubilization limit. There was also an increase with pH, so that, for example, at $\omega_o = 10$ the activity of the enzyme was nearly four-fold at pH 9 compared to its activity at pH 7.3.

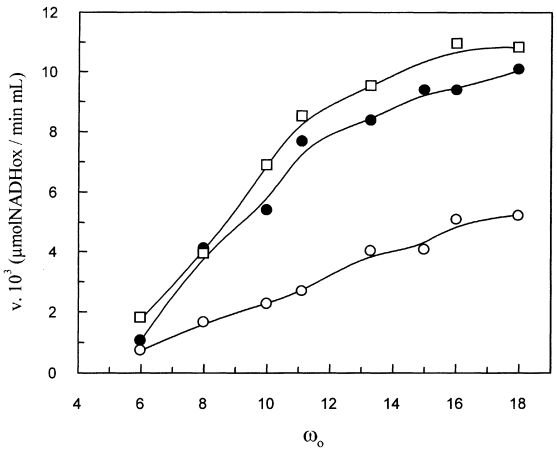

Fig. 1. Dependence of hMDH activity on the ω_o-value in CTAB/cyclohexane reverse micelles, at selected pH-values: (○) 50 mM sodium phosphate buffer, pH 7.3; (●) 50 mM sodium phosphate buffer, pH 8; (□) Tris/HCl buffer, pH 9.

3.2. Effect of water content on the ω_o-activity profile

Fig. 2 compares the specific activities of the hMDH in reverse micelles measured at a constant water concentration (open symbols), with those measured at a constant CTAB concentration (0.2 M; filled symbols). To change ω_o while maintaining the water content constant the same volume of water was injected into CTAB/cyclohexane/butanol solutions of different CTAB concentrations (0.1, 0.15, 0.2, 0.25 and 0.3 M). On the other hand, we changed the ω_o value at different volume of aqueous solution in a constant 0.2 M surfactant concentration. For both series of experiments, the specific activities (μmol NADHox/min ml mg of enzyme) show a maximum at different ω_o-values. This value is lower when the surfactant concentration is maintained constant. The specific activity the enzyme at a constant water concentration was maximal at $\omega_o = 9$ whereas at constant surfactant concentration

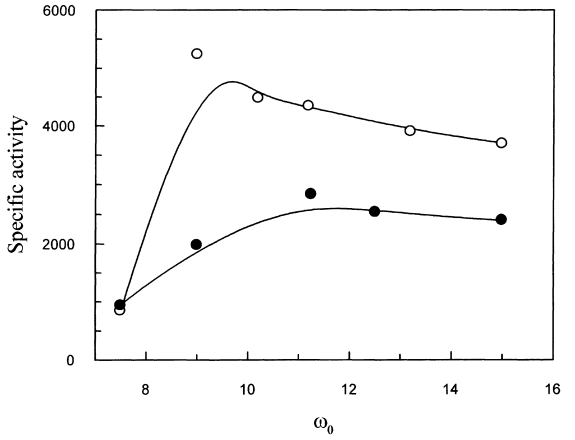

Fig. 2. Dependence of hMDH specific activity (units μmol NADHox/min ml mg of enzyme) on ω_o in CTAB/cyclohexane reverse micellar system, water amount constant at different concentrations of surfactant (0.1, 0.15, 0.2, 0.25 and 0.3 M CTAB (●)) or 0.2 M CTAB concentration constant (○).

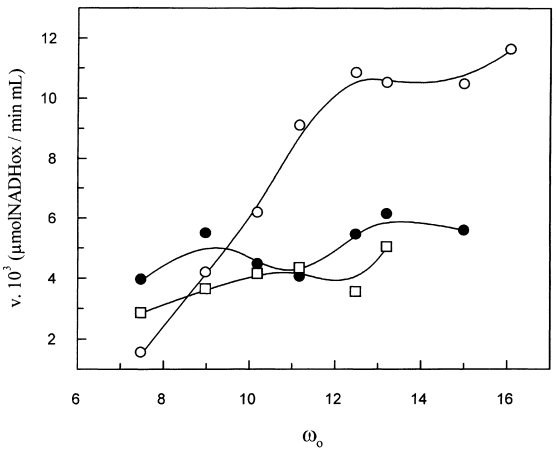

Fig. 3. Dependence of hMDH activity on ω_o in CTAB/cyclohexane reverse micellar system, aqueous buffer 50 mM sodium phosphate pH 7.3 containing 5 mM EDTA, 1 mM 2-mercaptoethanol and 0.05 M NaCl (○), 0.5 M NaCl (●) or 1 M NaCl (□). Concentrations: 5 mM NADH, 1 mM OAA and 0.0015 mg of enzyme.

it was maximal at $\omega_o = 11$ and it was nearly two-fold higher at constant water concentration.

3.3. Influence of salt concentration on ω_o-activity profile

The salt concentration of the buffer employed had profound effects both on the ω_o-activity profile as well as on the enzymatic activity. The ω_o-activity profiles at 1.0 and 0.5 M NaCl in phosphate buffer were very similar. In contrast, the ω_o-activity profile at low salt (0.05 M NaCl) showed a very steep increase reaching a plateau at ∼13 ω_o. The enzymatic activity at ω_o higher that 10 was the lowest at 1.0 M NaCl, it was slightly higher at 0.5 M NaCl but it was dramatically higher at 0.05 M NaCl. The enzymatic activity at ω_o of 13 was nearly three-fold higher than at 0.5 M NaCl and four-fold higher than at 1.0 M NaCl (Fig. 3).

A similar effect of salt concentration was found when the experiments were performed in Tris/HCl buffer instead of the phosphate buffer. Although the ω_o-activity profile was slightly different in the Tris/HCl buffer, nevertheless the enzymatic activity was substantially higher at 0.05 M NaCl than at 1.0 M NaCl (Fig. 4).

A similar influence of salt concentration on enzymatic activity was observed when the concentration of the enzyme was increased from 0.0015 to 0.0021 mg (data not shown).

3.4. Effect of surfactant concentration on the activity

Another factor that can influence enzymatic activity is the surfactant concentration. To examine the

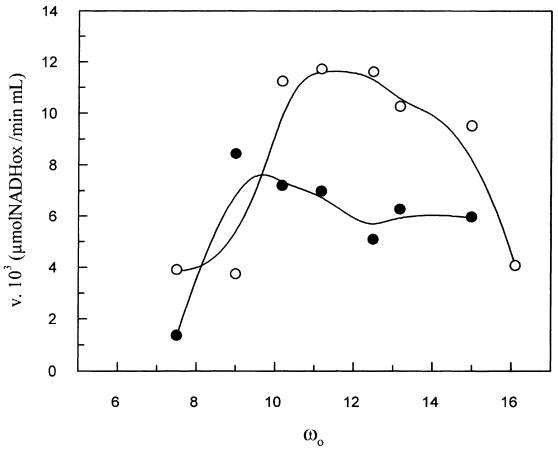

Fig. 4. Dependence of hMDH activity on ω_o in CTAB/cyclohexane reverse micellar system, aqueous buffer 100 mM Tris/HCl pH 7.3 containing 5 mM EDTA, 1 mM 2-mercaptoethanol and 0.05 M NaCl (○) or 1 M NaCl (●). Concentrations: 5 mM NADH, 1 mM OAA and 0.0015 mg of enzyme.

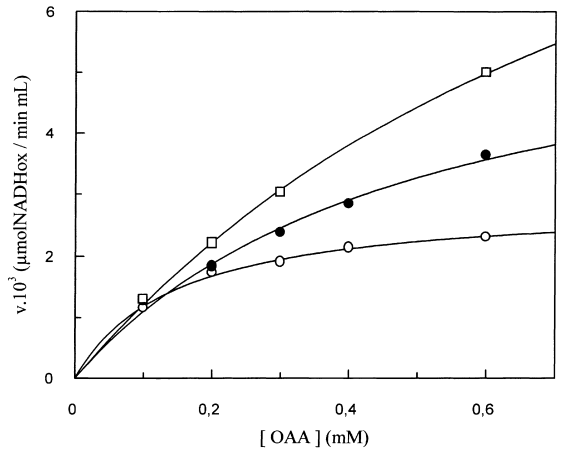

Fig. 5. Initial reaction rate of hMDH in reverse micellar media ($\omega_o = 10$) as a function of substrate concentration, at various CTAB concentrations: (○) 0.2 M; (●) 0.25 M; (□) 0.3 M CTAB. Buffer aqueous 50 mM sodium phosphate pH 7.3 containing 5 mM EDTA, 1 mM 2-mercaptoethanol and 1 M NaCl. (NADH) 2 mM and 0.009 mg of enzyme.

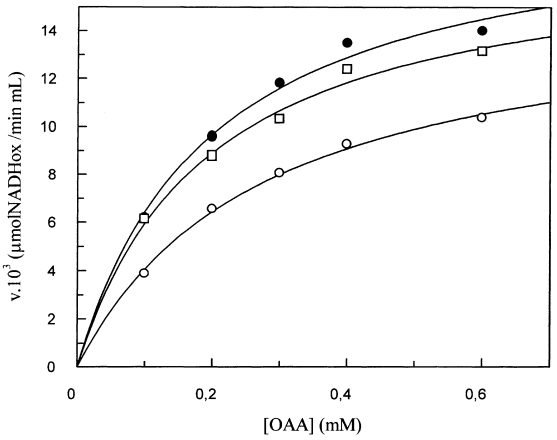

Fig. 6. Initial reaction rate of hMDH in reverse micellar media ($\omega_o = 15$) as a function of substrate concentration, at various CTAB concentrations: (○) 0.2 M; (●) 0.25 M; (□) 0.3 M CTAB. Buffer aqueous 50 mM sodium phosphate pH 7.3 containing 5 mM EDTA, 1 mM 2-mercaptoethanol and 1 M NaCl. (NADH) 2 mM and 0.009 mg of enzyme.

Table 1
Kinetic constants for hMDH in reverse micelles as a function of CTAB concentration, at $\omega_o = 10$[a]

[CTAB] (M)	V_{max} (10^3)	K_m^{OAA}
0.20	2.88 ± 0.08	0.14 ± 0.01
0.25	6.42 ± 0.79	0.48 ± 0.11
0.30	13.10 ± 0.94	0.98 ± 0.10

[a] Units of velocity, μmol NADHox/min ml and K_m^{OAA}, mM.

effects of varying surfactant concentration on the activity of hMDH we performed kinetic studies varying CTAB concentration, at two different ω_o. The results at $\omega_o = 10$ are shown in Fig. 5 and Table 1, and the results at $\omega_o = 15$ are shown in Fig. 6 and Table 2. Both experiments were obtained with the same enzyme concentration (0.009 mg per assay).

Table 2
Kinetic constants for hMDH in reverse micelles as a function of CTAB concentration, at $\omega_o = 15$[a]

[CTAB] (M)	V_{max} (10^3)	K_m^{OAA}
0.20	15.35 ± 0.67	0.28 ± 0.03
0.25	19.31 ± 1.25	0.20 ± 0.03
0.30	17.62 ± 1.01	0.20 ± 0.03

[a] Units of velocity (μmol NADHox/min ml) and K_m^{OAA} (mM).

The results showed that at lower ω_o ($\omega_o = 10$) both kinetic parameters V_{max} and K_m^{OAA} progressively increase with surfactant concentration indicating that the enzyme has a higher affinity for the substrate. When the micellar dimensions increase ($\omega_o = 15$) the enzymatic activity increases only at lower surfactant concentrations (up to 0.25 M CTAB) and decreases at higher surfactant concentration. Also, in contrast with the results obtained at $\omega_o = 10$, at the high ω_o ($\omega_o = 15$) the surfactant concentration does not result in a change in the affinity for the substrate. The affinity for the substrate (OAA) is similar at all surfactant concentrations studied (Table 2).

4. Discussion

Here, we examined the influence of various relevant factors on ω_o-activity profile of the extremely halophilic enzyme malate dehydrogenase (hMDH) in reverse micelles. We found that the behavior of this enzyme with increasing ω_o is to display an increased activity (Fig. 1) at all ω_o values examined. These results are in agreement with the previous suggestion that at higher water contents the media in the interior of reverse micelles behaves like a free solution, thus,

preserving a relatively intact conformation of the enzyme, and therefore, allowing high activity. In contrast, at lower water content the enzyme is entrapped in small micelles which could distort the structure of the enzyme lessening its activity [7]. The hydration of hMDH is only slightly higher than that of other non-halophilic proteins, yet NaCl "binding" is much higher than that of non-halophilic proteins [14]. The large number of acidic residues on the surface of hMDH permits enzyme molecules to bind more water than other proteins, and this may contribute to the creation of a larger hydration sphere that protects the enzyme from aggregating under high salt concentrations [15]. Despite the use of buffers of different pH, a similar trend was observed. However, when the micelles were formed with Tris/HCl buffer at pH 7.3, the ω_o-activity profile was different, showing the typical bell-shaped curve (see Fig. 2). These results indicate that the buffer component and pH not only affect the enzyme activity, but also affect the ω_o-activity profile. Some authors have proposed that the dimensions of the water pool at which catalysis is maximal coincide with the dimensions of the enzyme [10].

When we compared the ω_o-activity profile under conditions that changed the water content inside the micelles (Fig. 2, open symbols) with conditions that maintained the water content constant (in which the number of enzyme molecules in the water phase remained constant), we found that the values obtained were very close to each other (Fig. 2, closed symbols). These results could be explained by the observations of Bru and Walde [16] indicating that depending on the kinetic interpretation of the data, it is possible to obtain different profiles. These authors found that K_{cat} versus ω_o did not show a bell shape, whereas the plot of initial rate at saturating substrate concentration resulted in a bell shaped profile.

We also found that the salt concentration influences profoundly the ω_o-activity profile (Fig. 3). The influence of salt on hMDH in aqueous medium is known [17]. With a few exceptions, halophilic enzymes require a high salt concentration to remain stable. In molar NaCl and KCl solutions, hMDH molecules form particles that bind salt ions and water molecules. It has been calculated that under these conditions, one dimer is associated with about 4000 molecules of water and 500 molecules of salt [17]. We found that in reverse micelles of CTAB the enzyme is active when solubilized in a buffer at lower salt concentration. In contrast, most halophilic enzymes in aqueous medium display decreased or absent enzymatic activity, because at low salt concentration (<2 M NaCl) they likely become denatured. The reasons for our observation that hMDH retains its activity at lower salt concentration only when suspended in reverse micelles but not in aqueous medium, are not clear. The presence of salt may have different effects: salt may modify the properties of water, either favoring or limiting its dissolution by the micelles. Alternatively, electrostatic interactions and shielding of the charged groups of the CTAB molecule may also play a role; and may also influence the number of micelles [18]. However, the molecular mechanism that imparts hMDH the ability to function in lower salt concentration in reverse micelles is not known.

It is possible that a change of the buffer (Tris/HCl) should modify the association between salt and water molecules, and therefore, results in a different dependence activity versus ω_o (Fig. 4). It is also likely that the amount of water affects the interaction of the protein with the micelle wall when ligand concentration changes. These observations are in agreement with studies showing that the amount of water in contact with the enzyme controls the structural and catalytic characteristics of hMDH [14].

When we examined the effects of varying the enzyme concentration we found that at higher enzyme concentration the optimal ω_o value also increased (data not shown). This effect may be the result of a "higher protection" at high enzyme concentration, because a lower proportion of the enzyme may be inactivated during the solubilization.

We also showed that despite a slight change in the ω_o-activity profile, the enzymatic activity was higher at low salt concentration even when we employed a different buffer (Tris/HCl) as illustrated in Fig. 4. These results are in contrast with the observations of Pundak et al. [19] who found inactivation, dissociation, and unfolding of the enzyme (from the species *Halobacterium*) at NaCl concentrations below 2 M in aqueous media. We believe that the micellar medium protected the enzyme from denaturation and dissociation, thus, allowing the maintainance of high enzymatic activity.

Increasing surfactant concentration under conditions that maintain ω_o, substrate, and enzyme concentra-

tions constant, implies that there is a larger number of micelles, a smaller fractional occupancy of enzyme, and a localized decrease in the substrate concentrations within the water pool or at the micelle surface. The mechanisms of decrease of the activity under these conditions are not entirely apparent. However, it is likely that some of the inhibition can be simply attributed to reduced enzyme-substrate contact.

The results described here suggest the possibility of a biotechnological use of this enzyme in an alternative medium.

Acknowledgements

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References

[1] K. Martinek, A.V. Levashov, N.L. Klyachko, V.I. Pantin, I.V. Berezin, Biochem. Biophys. Acta 657 (1981) 277.
[2] P.L. Luisi, M. Giomini, M.P. Pileni, B.H. Robinson, Biochem. Biophys. Acta 947 (1988) 209.
[3] H. Ishikawa, K. Noda, T. Oka, J. Ferment. Bioeng. 70 (1990) 381.
[4] M.T. Patel, R. Nagarajan, A. Kilara, Biotechnol. Appl. Biochem. 22 (1995) 1.
[5] D. Han, P. Walde, P.L. Luisi, Biocatalysis 4 (1990) 153.
[6] K. Shiomori, M. Ishimura, Y. Baba, Y. Kawano, R. Kuboi, I. Komasawa, J. Ferment. Bioeng. 81 (1996) 143.
[7] Y.L. Khmelnitski, A. Van Hoek, C. Veeger, A.J.W.G. Visser, Eur. J. Biochem. 212 (1993) 63.
[8] R.O. Anarbaev, I.B. Elepov, O.I. Lavrik, Biochem. Biophys. Acta 1384 (1998) 315.
[9] F.C. Marhuenda-Egea, S. Piera-Velázquez, Q. Cadenas, E. Cadenas, Biocatal. Biotrans. 18 (2000) 201.
[10] A.V. Kabanov, A.V. Levashov, N.L. Klyachko, S.N. Namyotkin, A.V. Pshezhetsky, K. Martinek, J. Theoret. Biol. 133 (1988) 327.
[11] G. Chang, S. Shiao, Eur. J. Biochem. 220 (1993) 861.
[12] F. Rodriguez-Valera, F. Ruiz-Berraquero, A. Ramos-Comenzana, Can. J. Microbiol. 26 (1980) 1259.
[13] M. Mevarech, H. Eisenberg, E. Neumann, Biochemistry 16 (1977) 3781.
[14] H. Eisenberg, Biophys. Chem. 53 (1994) 57.
[15] O. Dym, M. Mevarech, J.L. Sussman, Science 267 (1995) 1344.
[16] R. Bru, P. Walde, Eur. J. Biochem. 199 (1991) 95.
[17] G. Zaccai, H. Eisenberg, TIBS 15 (1990) 333.
[18] E. Battistetl, P.L. Luisi, J. Col. Inter. Sci. 128 (1989) 7.
[19] S. Pundak, H. Aloni, H. Eisenberg, Eur. J. Biochem. 118 (1981) 471.