MICELLAR ENZYMOLOGY: SUPERACTIVITY OF ENZYMES IN REVERSED MICELLES OF SURFACTANTS SOLVATED BY WATER/ORGANIC COSOLVENT MIXTURES

Natalia L. KLYACHKO[a], Natalia G. BOGDANOVA[a], Andrei V. LEVASHOV[a] and Karel MARTINEK[b]

[a] *Department of Chemistry, Moscow State University, Moscow, Russia*
[b] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia*

Received April 16, 1991
Accepted June 24, 1991

Catalytic properties of α-chymotrypsin, peroxidase and laccase, dissolved in water-immiscible organic solvents by entrapping them into the reversed micelles of surfactants solvated by water/organic cosolvent (glycerol or 1,4- or 2,3-butanediol or dimethyl sulfoxide) mixtures, are studied. As micelle-forming surfactants, sodium salt of bis(2-ethylhexyl)sulfosuccinate (Aerosol OT) in n-octane or cetyltrimethylammonium bromide in n-octane/chloroform (1 : 1 by volume) mixture are used. The dependences of the catalytic activity on the surfactant solvation degree are bell-shaped. Maxima of the catalytic activity of enzymes solubilized in the micellar systems are observed at such optimum values of the surfactant solvation degree at which the size of micellar inner cavity and of the entrapped protein molecule is approximately equal. With decreasing content of water in the micellar media studied, the catalytic activity of the solubilized enzymes increases considerably, and is much (10—100 times) higher than in bulk aqueous buffers. In conclusion, possible mechanisms of the micellar effects are suggested.

Micellar enzymology[1] is a novel physicochemical line of approaches to problems of molecular biology. A general way how to affect the catalytic activity of enzymes entrapped into reversed micelles of surfactants in organic solvents is to vary the degree of surfactant hydration, i.e. molar ratio of H_2O/surf.; for review, see refs[1-6]. Recently[7] we have found that the catalytic activity of solubilized α-chymotrypsin increases considerably due to substituting a water-miscible cosolvent (glycerol) for water in the inner cavities of reversed micelles. For instance[7], the catalytic activity of α-chymotrypsin entrapped into Aerosol OT reversed micelles solvated with aqueous glycerol with the content of water being equal to about 5% (v/v) is approx. 10 times higher than in bulk water. In contrast to our micellar systems solvated by concentrated glycerol[7], the enzyme becomes completely inactive in a bulk medium of this cosolvent with the content of water being less than 30% (v/v) (ref.[8]).

The present work is aimed at a detailed study of the discovered[7] phenomenon, with various water-miscible cosolvents (1,4- and 2,3-butanediol, glycerol and di-

methyl sulfoxide) and different enzymes (peroxidase, laccase and α-chymotrypsin) being used for the construction of superactive biocatalytic micellar systems.

EXPERIMENTAL

Materials

α-Chymotrypsin was a Reanal reagent. The commercial preparation contained 60% of the active enzyme, according to the titration procedure[9]. Peroxidase of grade A was an Olaine plant (U.S.S.R.) preparation. The enzyme concentration was assayed spectrophotometrically[10] at 403 nm, the value of molar absorbance being equal to 91 000 $mol^{-1}\,l\,cm^{-1}$. Laccase from *Corriolus versicolor* was kindly provided by Dr G. Pepanyan from Armenia. Its purity was assayed spectrophotometrically[11] at 610 nm (molar absorbance 4 900 $mol^{-1}\,l\,cm^{-1}$.

N-Benzoyl-L-tyrosine *p*-nitroanilide was purchased from Sigma. Pyrogallol (Wako) was purified by vacuum sublimation. Hydrogen peroxide was a Reakhim (U.S.S.R.) reagent. Its concentration was measured[12] spectrophotometrically at 230 nm (molar absorbance 72·7 $mol^{-1}\,.\,l\,cm^{-1}$).

Bis(2-ethylhexyl)sulfosuccinate sodium salt (Aerosol OT) was purchased from Merck. According to IR data, the preparation contained 0·4 mol of water per mol of the surfactant. Cetyl trimethylammonium bromide (CTAB) (Chemapol) was purified by precipitation from alcohol solutions with diethyl ether. After keeping in vacuum over P_2O_5 for several days, the preparation contained 0·2 mol of water per mol of the surfactant. These values (0·4 and 0·2, respectively) were taken into account when the total amount of water in the micellar systems was calculated.

Octane (Reakhim) was purified according to the standard procedure[13]. Chloroform (Reakhim) was washed with water and distilled from P_2O_5 before use. Glycerol (Reakhim) was vacuum-distilled and kept over 0·4 nm molecular sieves. Dimethyl sulfoxide and 1,4- and 2,3-butanediols were used as received from Fluka.

Determination of Enzyme Activity

All kinetic measurements were performed on a Beckman 25 spectrophotometer under steady-state conditions at 25°C.

α-Chymotryptic Hydrolysis of N-*Benzoyl-*L-*Tyrosine p-Nitroanilide*

In bulk solutions: 5 μl of 5 to 20 mM N-benzoyl-L-tyrosine *p*-nitroanilide in a dioxane/acetonitrile mixture (1 : 1 by volume) were put into the spectrophotometer cell containing 1·4 ml of 50 mM Tris-HCl buffer (pH 8·0). A stock-solution (5 μl) of $3{\cdot}2\,.\,10^{-4}$ mM α-chymotrypsin in 50 mM Tris-glycine buffer (pH 8·0) was then added. For determination of the enzyme activity in water/organic cosolvent mixtures, see ref.[8].

In micellar media: 3 μl of 40 to 150 mM N-benzoyl-L-tyrosine *p*-nitroanilide in a dioxane/acetonitrile mixture (1 : 1 by volume), 0 to 30 μl of 50 mM Tris-HC, buffer (pH 8·0) and 0 to 30 μl of a water-miscible organic cosolvent were added to 2 ml of 0·1M Aerosol OT in octane or 0·1M CTAB in octane/chloroform (1 : 1 by volume) mixture. The reaction was initiated by addition of 1 to 5 μl of 1 to 10 mM α-chymotrypsin stock-solution in 50 mM Tris-glycine buffer (pH 8·0) under vigorous shaking.

For the *p*-nitroanilide substrate used, the pH-value chosen corresponded[14] to a pH-independent region of the enzymatic reaction rate. For the pH effects observed usually in α-chymotryptic catalysis see ref.[15].

The initial rate of α-chymotryptic hydrolysis of the *p*-nitroanilide substrate was followed spectrophotometrically at 380 nm by monitoring the appearance of *p*-nitroaniline. The molar absorbance of the product[14] was weakly dependent on both the degree of surfactant hydration and the ratio of water/organic cosolvent, and varied from 9 000 to 11 000 $mol^{-1}\,l\,cm^{-1}$. For bulk aqueous solution (pH 8·0, 50 mM Tris-HCl buffer), molar absorbance was 14 000 $mol^{-1}\,l\,.\,.\,cm^{-1}$. The values of catalytic rate constant (k_{cat}) of the enzymatic reaction were determined by using the Lineweaver–Burk plots as described elsewhere[15]. Some instructive examples are given in Fig. 1.

Peroxidase-Catalyzed Oxidation of Mesidine by Hydrogen Peroxide

To 2 ml of 0·1M Aerosol OT in octane, a solution of mesidine (5 μl, 0·1 to 0·5 mol l^{-1}) in 2,3-butanediol together with 0 to 30 μl of 20 mM acetate buffer (pH 5·0) were added under vigorous shaking. A solution of peroxidase (1 μl, 0·3 mmol l^{-1}) in 20 mM phosphate buffer (pH 5·0; the stock-solution was kept at pH 7) was then added. The reaction was initiated under vigorous shaking by addition of hydrogen peroxide (3 μl; 4 to 20 mmol l^{-1}) dissolved in 20 mM acetate buffer (pH 5·0). The pH-value chosen corresponded[14] to a pH-optimum of the enzymatic reaction rate.

The initial rate of the enzymatic reaction was followed spectrophotometrically by monitoring the appearance of the product, 2,6-dimethyl-4-(2,4,6-trimethyl)-anyl, at 490 nm with the value of molar absorbance being equal to 790 $mol^{-1}\,l\,cm^{-1}$.

The values of catalytic rate constant (k_{cat}) were evaluated from the dependence of the reaction rate on mesidine concentration (using the Lineweaver–Burk plots) under conditions[14] when the reaction rate is independent on the concentration of hydrogen peroxide.

Peroxidase-Catalyzed Oxidation of Pyrogallol by Hydrogen Peroxide

To 2 ml of 0·1M Aerosol OT in octane, pyrogallol (3 μl, 0·15 to 0·5 mol l^{-1}) dissolved in acetone, 0 to 30 μl of 2,3-butanediol and 0 to 30 μl of aqueous 50 mM phosphate buffer (pH 7·0) were added to achieve the desired values of both the degree of surfactant hydration and the ratio of water/2,3-butanediol. A solution of peroxidase (2 μl, 0·1 μmol l^{-1}) in 50 mM phosphate buffer (pH 7·0) was then added. The reaction was initiated by addition of hydrogen peroxide (2 μl, 4 to 20 mmol l^{-1}) dissolved in 50 mM phosphate buffer (pH 7·0), and its initial rate was followed spectrophotometrically by monitoring the appearance of the product, purpurogalline, at 420 nm, the value of molar absorbance being equal to 4 400 $mol^{-1}\,l\,cm^{-1}$.

For determination of the peroxidase activity in bulk aqueous buffer see ref.[16]. The pH-value chosen corresponded[16] to pH-independent region of the enzymatic reaction rate. The values of catalytic rate constant (k_{cat}) were calculated as described elsewhere[16].

Laccase-Catalyzed Oxidation of Pyrocatechol by Molecular Oxygen

To 1 ml of 0·1M CTAB in octane/chloroform mixture (1 : 1 by volume) containing 10 to 50 mM pyrocatechol, 0 to 60 μl of an organic cosolvent and 1 to 80 μl of 20 mM phosphate–acetate buffer (pH 3·5 – 5·5) were added to achieve the desired values of both the degree of surfactant hydration and the ratio of water/organic cosolvent. An aqueous solution of laccase (2 μl, 15 μmol l^{-1}) was then added under vigorous shaking.

The enzymatic activity was assayed[17] in the resulting optically transparent solution by spectrophotometric detection of the reaction product, *o*-benzoquinone, at 417 nm with the value of molar absorbance being equal to 520 $mol^{-1}\,l\,cm^{-1}$. For determination of the laccase activity in bulk aqueous buffer see ref.[17]. The values of catalytic rate constant (k_{cat}) were evaluated from the Lineweaver–Burk plots, and the pH-optimum k_{cat} values were determined for each composition of the reaction medium as described elsewhere[17].

RESULTS AND DISCUSSION

As before[1], as a criterion of the "true" catalytic activity of an entrapped enzyme, we have adopted a pH-independent value of the catalytic constant (k_{cat}), see Experimental, which is free from local effects of pH shift and substrate concentration

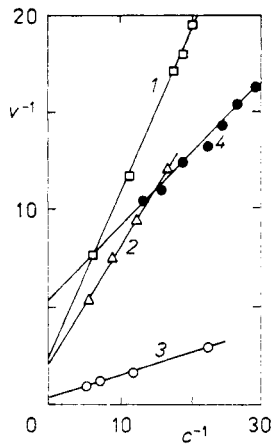


FIG. 1

Lineweaver–Burk plot for the initial steady-state rate of N-benzoyl-L-tyrosine *p*-nitroanilide hydrolysis catalyzed by α-chymotrypsin in 0·1M Aerosol OT–water/organic cosolvent mixture–octane: *1* hydrated reversed micelles (containing no organic cosolvent) at the optimum value of $[H_2O]/[surf]$ equal to 10·5 (cf. curves *1* in Figs 2 and 3); *2* and *3*, reversed micelles solvated by 48% (v/v) aqueous glycerol (at the optimum value of $[H_2O]/[surf]$ equal to 3·5, cf. curve *3* in Fig. 2) and 76% (v/v) aqueous 2,3-butanediol (at the optimum value of $[H_2O]/[surf]$ equal to 1·95, cf. curve *4* in Fig. 3), respectively; *4* data for α-chymotryptic catalysis in bulk water are presented for comparison. Experimental conditions: 25°C, pH 8·0 (0·05M Tris-HCl buffer), $1{\cdot}15 \cdot 10^{-6}$M α-chymotrypsin. *c* Concentration of substrate in mmol l^{-1}, *v* reaction rate in μmol l^{-1} s^{-1}

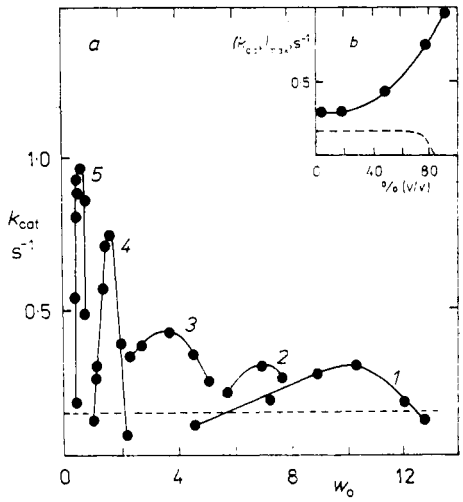


FIG. 2

α-Chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine *p*-nitroanilide in the Aerosol OT reversed micelles solvated by aqueous glycerol in octane. *a* w_o Water/Aerosol OT molar ratio, k_{cat} vs w_o profiles at different volume ratios of water/glycerol: *1* 100/0, *2* 81/19, *3* 52/48, *4* 24/76, *5* 10/90. Broken line shows the value of k_{cat} in bulk aqueous buffer (without the organic cosolvent), equal to 0·15 s^{-1}. *b* Maximum values of k_{cat} vs glycerol content % (v/v) in the water/organic cosolvent mixture entrapped into the reversed micelles. Broken line shows the k_{cat} values in bulk aqueous glycerol. Experimental conditions: 0·1M Aerosol OT, pH 8·0 (0·05M Tris-HCl buffer), 25°C

in micelles; for a review see refs[18,19]. In particular, the k_{cat} value, contrary to the apparent value of Michaelis constant, is not affected by the partitioning of reagents in the microheterogeneous (micellar) media[15,20]. Moreover, this value, measured during saturation of the enzyme with substrate, cannot be influenced by diffusional effects[18,21,22]. Some instructive Lineweaver–Burk relationships are given in Fig. 1; cf. also the plots of that kind published before[15–17].

Catalysis by α-Chymotrypsin

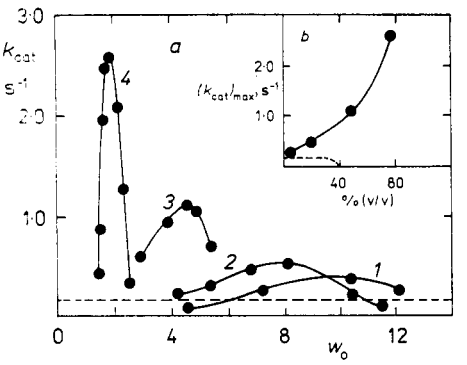
N-benzoyl-L-tyrosine *p*-nitroanilide is known[23] to be one of the specific substrates of α-chymotrypsin. We have found that not only in aqueous solution but in the systems of reversed micelles as well, this substrate does not undergo hydrolysis under the action of chymotrypsinogen, the structural analogue and precursor of the enzyme. Hence, the specific hydrolysis studied occurs at the active site of the enzyme, other functional groups of the protein globule being not involved.

Our choice of one of the anilide substrates is determined by the fact that the limiting step of their enzymatic hydrolysis is the acylation of the enzyme[24,25]. It means that the apparent value of catalytic rate constant (k_{cat}) cannot be affected by organic cosolvent as an additional nucleophile[24–26].

The experimental data are given in Figs 2–5. For the anilide substrate used, the dependences of the catalytic activity on the molar ratio of water to surfactant concentration are bell-shaped (see curves *1*), as has been earlier[1–6] found for other (ester) substrates of the enzyme. Addition of a water-miscible organic cosolvent into the reaction mixture (curves *2–5*) induces remarkable changes of the k_{cat} vs $[H_2O]/[surf.]$ profiles regardless of the nature of both cosolvent (glycerol in Figs 2

FIG. 3

α-Chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine *p*-nitroanilide in the Aerosol OT reversed micelles solvated by aqueous 2,3-butanediol in octane. *a* w_o Water/Aerosol OT molar ratio, k_{cat} vs w_o profiles at different volume ratios of water/2,3-butanediol: *1* 100/0, *2* 81/19, *3* 52/48, *4* 24/76. Broken line shows the value of k_{cat} in bulk aqueous buffer (without the organic cosolvent), equal to 0·15 s^{-1}. *b* Maximum values of k_{cat} (from Fig. 3*a*) vs 2,3-butanediol content % (v/v) in the water/organic cosolvent mixture entrapped into the reversed micelles. Broken line shows the values of k_{cat} in bulk aqueous 2,3-butanediol. For experimental conditions see the legend to Fig. 2



and 4, and 2,3-butanediol in Fig. 3, and dimethyl sulfoxide in Fig. 5) and surfactant (Aerosol OT, anionic surfactant, in Figs 2–3, and CTAB, cationic surfactant, in Figs 4–5).

What are the main effects? First, with increasing content of organic cosolvent, the profiles studied shift to the region of low hydration degree, i.e. the optimum content of water dramatically decreases (Figs 2*a*–5*a*). In other words, with increasing proportion of organic cosolvent the enzyme requires lower and lower quantity of

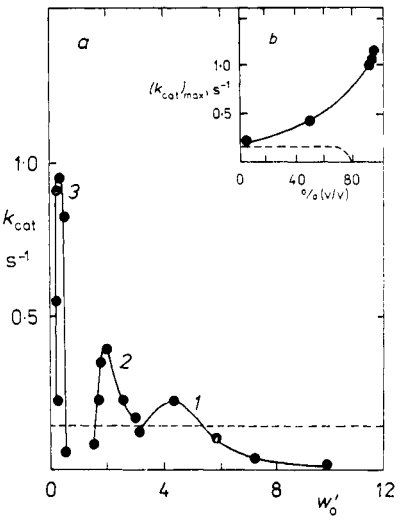


FIG. 4

α-Chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine *p*-nitroanilide in the CTAB reversed micelles solvated by aqueous glycerol in octane/chloroform mixture (1 : 1, by volume). *a* w_o' Water/CTAB molar ratio, k_{cat} vs $[H_2O]/[surf]$ profiles at different volume ratios of water/glycerol: *1* 100/0, *2* 51/49, *3* 8/92. Broken line shows the value of k_{cat} in bulk aqueous buffer (without the organic cosolvent), equal to 0·15 s^{-1}. *b* Maximum values of k_{cat} (from Fig. 4*a*) vs glycerol content % (v/v) in the water/organic cosolvent mixture entrapped into the reversed micelles. Broken line shows the values of k_{cat} in bulk aqueous glycerol. Experimental conditions: 0·1M CTAB, pH 8·0 (0·05M Tris), 25°C

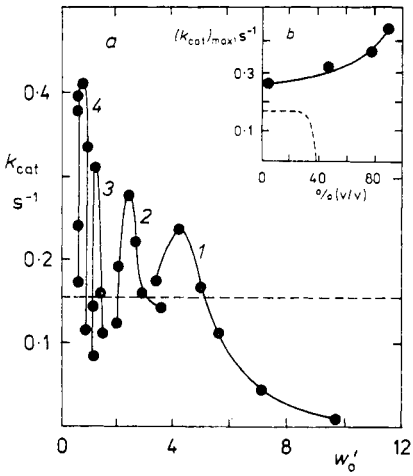


FIG. 5

α-Chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine *p*-nitroanilide in the CTAB reversed micelles solvated by aqueous dimethylsulfoxide in octane/chloroform mixture (1 : 1). *a* w_o' Water/CTAB molar ratio, k_{cat} values vs w_o' profiles at different volume ratios of water/dimethyl sulfoxide: *1* 100/0, *2* 51/49, *3* 22/78, *4* 13/87. Broken line shows the k_{cat} value in bulk aqueous buffer (without the organic cosolvent), equal to 0·15 s^{-1}. *b* Maximum values of k_{cat} (from Fig. 5*a*) vs dimethyl sulfoxide % (v/v) content in the water/organic cosolvent mixture entrapped into the reversed micelles. Broken line shows the values of k_{cat} in bulk aqueous dimethyl sulfoxide. For experimental conditions, see the legend to Fig. 4

water to manifest its maximum activity. Second, there is a considerable increase in the maximum activity as the content of organic cosolvent in the inner cavity of micelles increases (Figs 2*b*–5*b*).

These two results mean that the enzyme perfectly works under more and more "dry conditions". In contrast to the micellar media studied, the enzyme dissolved in bulk water/organic cosolvent mixtures[8] loses completely its catalytic activity (broken lines in Figs 2*b*–5*b*).

Catalysis by Peroxidase

Peroxidase catalysis was studied using as example the oxidation of two substrates, pyrogallol[27] and mesidine[28], by hydrogen peroxide. Each of the substrates gives only one, well-characterized product[29]. The compounds chosen are typical representatives of two classes of peroxidase substrates, phenols and amines. The enzymatic reactions were carried out in two micellar media: Aerosol OT–aqueous glycerol–octane or Aerosol OT–aqueous 2,3-butanediol–octane.

Despite the nature both of substrate and of reaction medium (organic cosolvent), the peroxidase activity varies with the degree of surfactant hydration in the same manner as in the above α-chymotrypsin case. An example of the experimental data obtained is given in Fig. 6. As seen, addition of 2,3-butanediol to the micellar system, first, induces a shift of the k_{cat} vs $[H_2O]/[surf]$ profiles to the region of low hydration degree, and, second, increases the catalytic activity.

Catalysis by Laccase

1,4-Butanediol induces the same changes of the k_{cat} vs $[H_2O]/[surf]$ profiles as

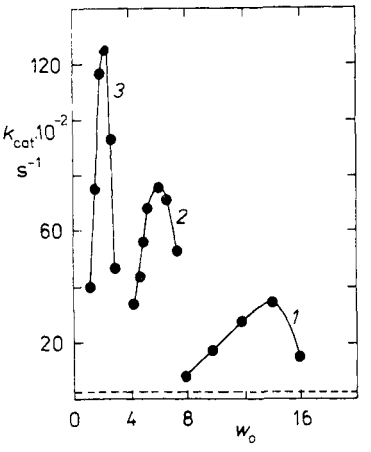


FIG. 6

Peroxidase-catalyzed oxidation of pyrogallol in the Aerosol OT reversed micelles solvated by aqueous 2,3-butanediol in octane. w_o Water/Aerosol OT molar ratio, k_{cat} vs w_o profiles at different volume ratios of water/2,3-butanediol: *1* 100/0, *2* 52/48, *3* 24/76. Experimental conditions: 0·1M Aerosol OT, pH 7·0 (0·05M phosphate buffer), 25°C. Broken line shows the k_{cat} value in bulk aqueous buffer (without the organic cosolvent), equal to 150 s^{-1}

in the above two cases of α-chymotrypsin and peroxidase. First, with increasing content of 1,4-butanediol, the profiles studied shift to the region of low hydration degree (Fig. 7). Second, an increase in the concentration of 1,4-butanediol up to 76% (v/v) is accompanied by a more than three-fold increase in the catalytic activity of the enzyme (Fig. 7, cf. curves *1* and *3*).

However, the replacement of water in the inner cavities of micelles for other cosolvents decreases the catalytic activity four-fold (Fig. 7, curves *4* and *5*). The anomalous behavior of 2,3-butanediol (and glycerol as well, data not shown) probably arises from specific interactions between these cosolvents (with neighbouring OH-groups in their molecules) and copper ions in the active site of laccase[11].

Possible Mechanisms of the Micellar Effects

(i) Why are the k_{cat} versus $[H_2O]/[surf]$ profiles bell-shaped? The problem is possible to be rationalized in terms of our concept[30,31] assuming that solubilized enzymes display the maximum values of their catalytic activity when the size of the molecule of the enzyme entrapped corresponds to the inner diameter of the empty micelle. Let us analyze this assumption in more detail. As shown for Aerosol OT in hydrocarbons[32], the hydration degree is a value determining the size of the inner water-cavity of reversed micelles (at constant surfactant concentration); for a review see refs[18,19,33]. In the present work, the highest catalytic activity in the Aerosol

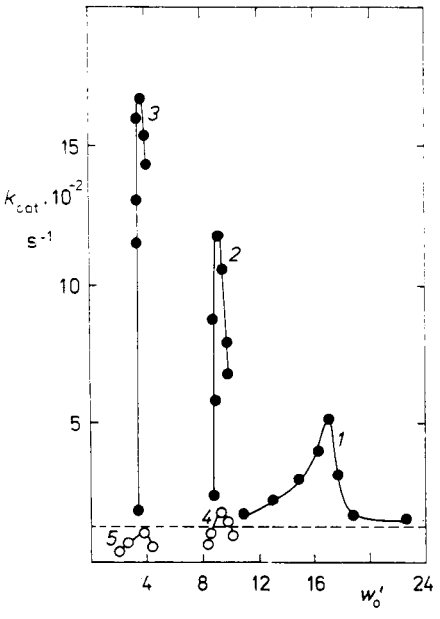


FIG. 7

Laccase-catalyzed oxidation of pyrocatechol in the CTAB reversed micelles solvated by aqueous 1,4-butanediol (●) or aqueous 2,3-butanediol (○) in octane/chloroform mixture (1 : 1 v/v). w_o' Water/CTAB molar ratio, k_{cat} vs w_o' profiles at different volume ratios, of water/butanediol: *1* 100/0, *2* and *4* 51/49, *3* and *5* 22/78. Experimental conditions: 0·1M CTAB, 20 mM phosphate–acetate buffer, pH 3·5—5·5 (the pH-optimum k_{cat} values were determined for each composition of the reaction medium), 25°C. Broken line shows the value of k_{cat} in bulk aqueous buffer (with no organic cosolvent), equal to 130 s^{-1} at pH 4·3 (20 mM phosphate–acetate buffer) and 25°C

OT–water–octane medium is displayed when the degree of surfactant hydration is approx. 10·5 (see curves *1* in Figs 2*a*–3*a*) and approx. 14 (see curve *1* in Fig. 6) for α-chymotrypsin and peroxidase, respectively. Indeed, under these hydration conditions, the inner radia of the empty Aerosol OT reversed micelles (equal to 2·0 and 2·4 nm, respectively[32,33]) are in good accord with the size of the enzyme molecules entrapped: the ellipsoidal dimensions of α-chymotrypsin molecule (4·0 × 4·0 × × 5·0 nm)[34,35] are possible to be approximated[33] by the sphere of equal volume with a radius of about 2·15 nm; the radius of peroxidase molecule[36] is equal to 2·5 nm. The two enzymes are the first ones studied in 1977 in terms of micellar enzymology using synthetic surfactants[37,38]; cf. historical assay[1]. Until now, the coincidence of the optimum diameter of the inner cavity of hydrated micelles (containing no organic cosolvent) with the molecular size of the entrapped protein molecules has been observed for about twenty enzymes[4–6,*].

At lower hydration degrees, the empty micelles are too small for the whole enzyme molecule to be entrapped. Thus, the molecule of a solubilized enzyme forms its "own micelle" so as the surfactant monolayer might protect the protein globule surface from contact with organic solvent[33,39]. It is possible, however, that some segments of the enzyme molecule (including the active centre) can be partially exposed to organic solvent[40]. In principle, this can be accompanied by conformational changes in the protein globule, causing, for instance, reduction in catalytic activity. Such unfavourable conformational changes can also result from partial protein dehydration at low water content in the micellar medium[30]. Furthermore, the hydrolytic reactions (for instance, α-chymotryptic catalysis) can be decelerated by a lower concentration of "free" water[30].

At higher hydration degrees, when the radius of the inner micellar cavity exceeds the size of enzyme molecule, enzyme activity usually decreases to a greater or lesser

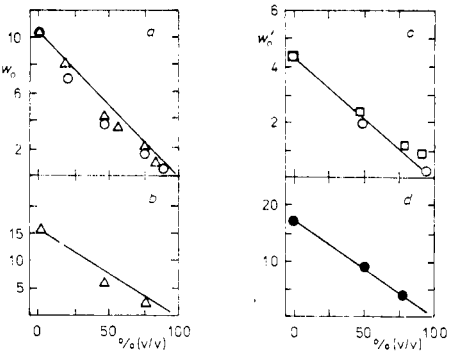


FIG. 8

The optimum values of $[H_2O]/[surf]$ molar ratio (cf. Fig. 2 as an example) vs the corresponding content of cosolvent % (v/v) in the water/organic mixtures used for the solvating of micellar media: ○ glycerol, ● 1,4-butanediol, △ 2,3-butanediol, and □ DMSO. Surfactant: *a*, *b* Aerosol OT, *c*, *d* CTAB. Micellar media: *a*, *c*, chymotrypsin, *b* peroxidase, *d* laccase. For experimental conditions see the legend to Figs 2–7

* As an instructive example, see recent data[74] on kinetics of soybean lipoxygenase reaction in hydrated reversed micelles.

extent; for a review see refs[4-6]. It is quite possible that enzymes capable of multi-point interaction with the micellar interface, when entrapped in a large micelle, can unfold (denature)[19].

Of course, there may be other molecular mechanisms that can explain the enzyme catalytic activity as a bell-shaped function of micelle dimensions. Nevertheless, despite the possible variety and complexity of these mechanisms[19], the k_{cat} value is maximum in "optimum micelles" (containing no organic cosolvent) where the mean radius of their inner cavities coincides with the molecular size of the solubilized enzyme[4-6].

(ii) Is the principle of geometric fit for the micellar systems solvated by water-miscible organic cosolvents valid? In order to answer it, let us pay attention to the fact that (Fig. 8) the optimum degree of surfactant hydration (when the catalytic activity is maximum) and the content of organic cosolvent are inversely proportional quantities. As seen in Fig. 8, this is valid for all biocatalytic micellar systems studied (Figs 2–7). What does it mean? On condition that the micellar system is monodisperse*, the volume of inner cavities inside the optimum micelles solvated by water/organic mixtures is evidently constant – regardless of varying the value of molar ratio "water/organic cosolvent".

In order to analyze this question in more detail, the value of inner cavity radius (r_{in}) should be evaluated. It can be done in terms of the equation:

$$r_{in} = 3V_{in}/S_{in}\,, \tag{1}$$

where $V_{in} = 4\pi r_{in}^3/3$ and $S_{in} = 4\pi r_{in}^2$ are the volume and the surface, respectively, of aqueous (or water/organic cosolvent) drop inside the reversed micelles. If the micellar system is close to monodispersed one, the former value can be expressed as

$$V_{in} = V_t n/CN\,, \tag{2}$$

where V_t is the total volume (in cm^3) of water/organic cosolvent mixture per 1 l of the micellar medium, C molar concentration of surfactant, N Avogadro number, and n is the aggregation number. As to the latter value,

$$S_{in} = nf\,, \tag{3}$$

where f is the area of surfactant molecule on the surface of the inner cavity of the micelle. Combination of Eqs (1)–(3) gives

$$r_{in} = 3V_t/CNf\,. \tag{4}$$

The values V_t and C may be drawn from our experimental data, and the f parameter.

* This is probably valid for Aerosol OT micellar systems[32,41-48]; for review see ref.[19]

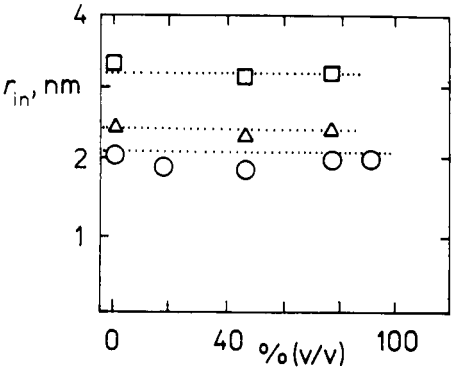
is known from literature for the Aerosol OT micelles solvated by water[32,33] and pure (dry) glycerol[47,48]. For the solvating mixtures of water/glycerol type, we have accepted that the f parameter depends in a linear manner of the content of organic cosolvent.

Figure 9 shows a dependence of the r_{in} values evaluated using Eq. (*4*), on the content of glycerol in the water/organic cosolvent mixtures used for the solvation of micelles. Dotted lines in Fig. 9 show the size of α-chymotrypsin[33–35] and peroxidase[36] molecules entrapped, see section (i) above. The data[49] are also given for penicillin acylase. For all three enzymes (see Fig. 9), the size of their molecules (dotted lines) is actually equal to the dimensions of the inner cavities of solvated micelles. It should be stressed, however, that such an approximate coincidence takes place only under the optimum conditions of surfactant hydration (when the solubilized enzymes just display the maxima of their catalytic activity; cf. Fig. 2, for instance).

(iii) Superactivity of the enzymatic catalysis in reversed micelles. We suppose that the superactivity observed (Figs 2–7) is due to the relatively high rigidity of the surfactant shell fit close to the molecule of the solubilized enzymes (Figs 9 and 10). Such a close-fitting cage of surfactant molecules may function as a buffer (or damper) of excessive spontaneous fluctuations that usually destroy (or disturb) the catalytical conformations of the active centers of enzymes both in bulk aqueous[50,51] and in bulk water/organic[8,52] solutions. In other words, under conditions when a complementarity between the micellar cavity and the enzyme molecule entrapped is reached (Fig. 9) the surrounding surfactant matrix can tightly "squeeze" the latter, thereby "freezing" its rotational and vibrational motions which could lead to the fixation of the most catalytically active conformation (Fig. 10). In fact, the interface tension should be considered main driving force that stimulates the considerable enhancement observed (Figs 2–7) in enzyme action. Such a "micromechanical" effect

FIG. 9

The value of inner cavity radius (nm) for the Aerosol OT micelles solvated by aqueous glycerol in n-octane (evaluated in terms of Eq. (*4*) for the optimum values of surfactant hydration degree when the values of catalytic activity are maximum; for instance, cf. Fig. 2 for α-chymotrypsin) vs the content of glycerol (% (v/v)) in the water/organic cosolvent mixtures used for the solvating of micelles. Dotted lines show the size of enzyme molecules entrapped □ penicillin acylase, △ peroxidase, ○ chymotrypsin. For references see the text



on the catalytic activity was supposed before[4,6] to be valid for functioning enzymes entrapped into the reversed micelles solvated by water (containing no organic cosolvent).

The utilization of organic cosolvents instead of water in the inner cavities of micelles makes the micellar surface still stronger: the half-time of one molecule of surfactant in one micelle increases[47,48]. That is why an increase in the concentration of cosolvents is accompanied by increasing catalytic activity, as shown in Figs 2–7.

In order to support this hypothesis in detail, the rotational frequency of a spin label in the active center of solubilized enzymes has to be investigated in the future – as a function of the cosolvents concentration. Preliminary results[53] recently reviewed[4-6] are in good accord with all what has been said above.

CONCLUSIONS

Four points should be emphasized as conclusion. First, a prevailing role of geometric fit (compared with possible effects of local medium) discovered in the reversed micellar media, should manifest itself upon functioning enzymes in biomembranes as well. A relation of micellar enzymology to membranology has been recently[1,4]

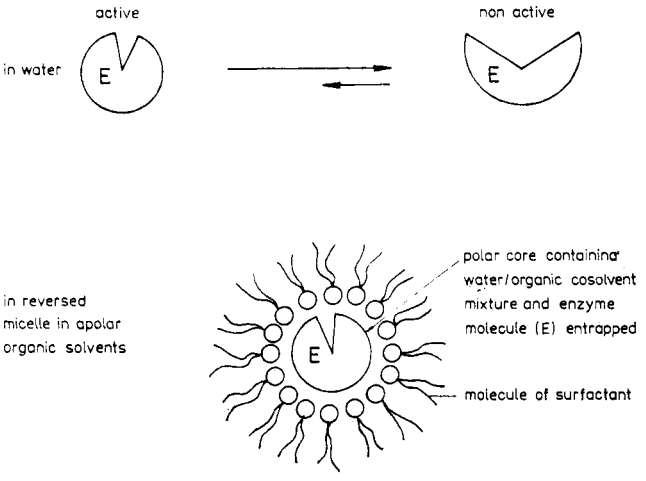


FIG. 10

Scheme of elimination of spontaneous fluctuations of enzyme (E) structure inside the reversed micelle, i.e. the fluctuations which usually disturb the catalytically active conformation in bulk solutions. Under hydration conditions when a complementarity between the micellar cavity and the enzyme molecule (E) entrapped is reached (cf. Fig. 9) the surrounding surfactant matrix tightly "squeezes" the latter, thereby "freezing" its rotational and vibrational motions which could lead to the fixation of the most catalytically active conformation

overviewed. In particular, such a "mechanical steering" of enzyme action (as outlined in Fig. 10, with the interface tension being main driving force) should function in a "metamorphic mosaic" model of biomembranes[55] we assume. At any rate, so-called lipidic particles, representing "reversed micelles" sandwiched between monolayers of lipid bilayer[55-61], may involve a mechanical mode of enzyme regulation like this.

Second, there are known other mechanical devices to "steer" (to regulate) enzymatic processes at the molecular level[62,63], for instance, by mechanical compression of a membrane with gel structure[64]. In contrast to them, the micellar mode of enzyme regulation (with the surfactant shell fit close to the molecule of biocatalyst entrapped) gives a unique chance to attain an enhancement of biocatalysis (its superactivity) compared with the level in bulk water.

Third, with using water-miscible organic cosolvents for solvating micellar media, it may be possible to create almost "dry" (anhydrous) conditions under which, nevertheless, the solubilized enzymes display high level of their catalytic activity. To demonstrate this, let us analyze in more detail the data obtained, for instance, for α-chymotryptic catalysis in CTAB micelles solvated by aqueous 92% (v/v) glycerol (Fig. 4, curve *3*). As seen, the optimum degree of surfactant hydration is approx. 0·4 with the catalytic activity exceeding considerably that observed in bulk water (dashed line). This value of $[H_2O]/[CTAB]$ molar ratio (being equal to 0·4) corresponds to about 60 molecules of water in one micelle, if the aggregation number for the CTAB micelles solvated by glycerol[47,48] is approx. 140, and, on entrapment of α-chymotrypsin molecule into a reversed micelle, the values of its aggregation number and hydration degree remain unchanged[33,54]; for review see refs[4,5]. This implies that, in micellar medium, the solubilized enzyme displaying a superactivity, is surrounded by only several dozens of water molecules per molecule of the protein, with the total concentration of H_2O in the whole system being less than 0·1% (v/v). Obviously, this result is of fundamental importance for enzyme membranology.

Fourth, applied biochemistry is keen on the retention of enzymatic function under such extreme (anhydrous) conditions[65-72]. Reversed micellar media have gained credence in analytical chemistry, fineorganic synthesis, bioconversion of energy, etc.; for some significant examples see ref.[6]. Recently, physicochemical principles for the construction of biocatalytic systems with a low content of water have been critically reviewed[73]. The reversed micellar approach studied not only provides another technique for enzyme engineering in non-aqueous media but gives a unique chance for the regulation of biocatalysis increasing considerably its activity (Figs 2–7) which is hardly accessible by other means[73].

REFERENCES

1. Martinek K., Levashov A. V., Klyachko N. L., Khmelnitsky Yu. L., Berezin I. V.: Eur. J. Biochem. *155*, 453 (1986).
2. Luisi P. L., Magid L. J.: CRC Crit. Rev. Biochem. *20*, 409 (1986).
3. Luisi P. L., Giomini M., Pileni M.-P., Robinson B. H.: Biochim. Biophys. Acta *947*, 209 (1988).
4. Martinek K., Klyachko N. L., Khmelnitsky Yu. L., Levashov A. V.: Biochim. Biophys. Acta *981*, 161 (1989).
5. Khmelnitsky Yu. L., Kabanov A. V., Klyachko N. L., Levashov A. V., Martinek K. in: *Structure and Reactivity in Reversed Micelles* (M.-P. Pileni, Ed.), p. 230. Elsevier, Amsterdam 1989.
6. Martinek K.: Biochem. Int. *18*, 871 (1989).
7. Klyachko N. L., Bogdanova N. G., Levashov A. V., Martinek K.: Dokl. Akad. Nauk SSSR *297*, 483 (1987).
8. Mozhaev V. V., Khmelnitsky Yu. L., Sergeeva M. V., Belova A. B., Klyachko N. L., Levashov A. V., Martinek K.: Eur. J. Biochem. *184*, 597 (1989).
9. Schonbaum G. R., Zerner B., Bender M. L.: J. Biol. Chem. *236*, 2930 (1961).
10. Maethly A. C.: Methods Enzymol. *2*, 801 (1953).
11. Reinhammar B. in: *Copper Proteins and Copper Enzymes* (R. Lontie, Ed.), Vol. 3, p. 1. CRC Press, Florida 1984.
12. George P.: Biochem. J. *54*, 267 (1953).
13. Martinek K., Khmelnitsky Yu. L., Levashov A. V., Klyachko N. L., Semenov A. N., Berezin I. V.: Dokl. Akad. Nauk SSSR *256*, 1423 (1981); Engl. Ed. *256*, 143 (1981).
14. Bogdanova N. G.: *Thesis*. Chemistry Department, Moscow State University, Moscow 1989.
15. Martinek K., Levashov A. V., Klyachko N. I., Pantin V. I., Berezin I. V.: Biochim. Biophys. Acta *657*, 277 (1981).
16. Klyachko N. L., Levashov A. V., Martinek K.: Mol. Biol. (Moscow) *18*, 1019 (1984); Engl. Ed. *18*, 830 (1984).
17. Pshezhetsky A. V., Klyachko N. L., Levashov A. V., Martinek K.: Biocatalysis *4*, 185 (1990).
18. Khmelnitsky Yu. I., Levashov A. V., Klyachko N. L., Martinek K.: Usp. Khim. *53*, 545 (1984); Engl. Ed. *53*, 319 (1984).
19. Kabanov A. V., Levashov A. V., Klyachko N. L., Martinek K.: J. Theor. Biol. *133*, 327 (1988).
20. Khmelnitsky Yu. L., Neverova I. N., Grinberg V. Ya., Levashov A. V., Martinek K.: Eur. J. Biochem. *190*, 155 (1990).
21. Berezin I. V., Klibanov A. M., Martinek K.: Usp. Khim. *44*, 17 (1975).
22. Goldstein L.: Methods Enzymol. *44*, 397 (1976).
23. Bundy H. F.: Arch. Biochem. Biophys. *102*, 416 (1963).
24. Berezin I. V., Martinek K.: Fizikokhimicheskie osnovy fermentativnogo kataliza. Vysshaya shkola, Moskva (1977).
25. Fersht A.: *Enzyme Structure and Mechanism*. Freeman, San Francisco 1977.
26. Dorovska-Taran V., Momtcheva R., Martinek K.: Biochim. Biophys. Acta *702*, 37 (1982).
27. Dure L. S., Cormier M. J.: J. Biol. Chem. *239*, 2351 (1964).
28. Brill A. S., Veinryb I.: Biochemistry *6*, 3528 (1967).
29. Saunders B. C., Holwes-Siedle A. G., Stark B. R.: *Peroxidase*. Butterworths, London 1964.
30. Levashov A. V., Klyachko N. L., Martinek K.: Bioorg. Khim. *7*, 670 (1981).
31. Belonogova O. V., Likhtenshtein G. I., Levashov A. V., Klyachko N. L., Khmelnitsky Yu. L., Martinek K.: Biokhimiya *48*, 379; Engl. Ed. *48*, 330 (1983).

32. Eicke M.-F., Rehak J.: Helv. Chim. Acta *59*, 2883 (1976).
33. Levashov A. V., Khmelnitsky Yu. L., Klyachko N. L., Martinek K.: J. Colloid Interface Sci. *88*, 444 (1982).
34. Squire P. O., Himmel M. E.: Arch. Biochem. Biophys. *196*, 165 (1979).
35. Blow D. M. in: *The Enzymes* (P. D. Boyer, Ed.), 3rd ed., Vol. 3, p. 185. Academic Press, New York 1971.
36. Clementi F., Palade C. E.: J. Cell Biol. *41*, 33 (1969).
37. Martinek K., Levashov A. V., Klyachko N. L., Berezin I. V.: Dokl. Akad. Nauk SSSR *236*, 920 (1977); Engl. Ed. *236*, 951 (1978).
38. Luisi P. L., Henninger F., Joppich M., Dossena A., Casnatti G.: Biochem. Biophys. Res. Commun. *74*, 1384 (1977).
39. Menger F. M., Yamada K.: J. Am. Chem. Soc. *101*, 6731 (1979).
40. Luisi P. I., Bonner F. J., Pellegrini A., Wiget P., Wolf R.: Helv. Chim. Acta *62*, 740 (1979).
41. Zulauf M., Eicke H.-F.: J. Phys. Chem. *83*, 480 (1979).
42. Fletcher P. D. I., Howe A. M., Perrins N. M., Robinson B. H., Toprakcioglu C., Dore J. C. in: *Surfactants in Solution* (K. L. Mittal and B. Lindmann, Eds), Vol. 2, p. 1745. Plenum, New York 1984.
43. Robinson B. H., Toprakcioglu C., Dore J. C., Chieux P.: J. Chem. Soc., Faraday Trans. 1 *80*, 13 (1984).
44. Kotlarchyk M., Chen S.-H., Huang J. S.: Phys. Chem. *86*, 3273 (1982).
45. Eicke H.-F., Kubic R., Hasse R., Zschokke I. in: *Surfactants in Solution* (K. L. Mittal and B. Lindmann, Eds), p. 1533. Plenum, New York 1984.
46. Clarke J. H. R., Nicholson J. D., Regan K. N.: J. Chem. Soc., Faraday Trans. 1 *81*, 1173 (1985).
47. Fletcher P. D. I., Galal M. F., Robinson B. H.: J. Chem. Soc., Faraday Trans. *80*, 3507 (1984).
48. Fletcher P. D. I., Galal M. F., Robinson B. H.: J. Chem. Soc., Faraday Trans. 1 *81*, 2053 (1985).
49. Klyachko N. L.: *Thesis*. Chemistry Department, Moscow State University, Moscow 1990.
50. Martinek K., Mozhaev V. V.: Adv. Enzymol. *57*, 179 (1985).
51. Mozhaev V. V., Martinek K.: CRC Crit. Rev. Biochem. *23*, 235 (1988).
52. Mozhaev V. V., Sergeeva M. V., Belova A. B., Khmelnitsky Yu. L., Martinek K.: Biotechnol. Bioeng. *35*, 653 (1990).
53. Klyachko N. L., Bogdanova N. G., Koltover V., Levashov A. V., Martinek K.: Biokhimiya *54*, 1224 (1989).
54. Shapiro Yu. E., Budanov N. A., Levashov A. V., Klyachko N. L., Martinek K.: Collect. Czech. Chem. Commun. *54*, 1126 (1989).
55. Cullis P. R., De Kruijff B., Hope M. J., Nayar R., Schmid S. L.: Can. J. Biochem. *58*, 1091 (1980).
56. Cullis P. R., De Kruijff B., Hope M. J., Verkleij A. J., Nayar R., Bally B. in: *Membrane Fluidity in Biology* (R. C. Alois, Ed.), p. 39. Academic Press, New York 1983.
57. De Kruijff B., Cullis P. R., Verkleij A. J., Hope M. J., Taraschi T. F. in: *The Enzymes of Biological Membranes* (A. N. Martonosi, Ed.), 2nd ed., Vol. 1, p. 131. Plenum, New York 1985.
58. Cullis P. R., Hope M. J., Tilcock C. P. S.: Chem. Phys. Lipids *40*, 127 (1986).
59. Stier A., Finch S. A. E., Börsterling B.: FEBS Lett. *91*, 109 (1978).
60. Murphy D. J.: FEBS Lett. *150*, 19 (1982).
61. Stier A., Finch S. A. E., Greinert R., Hohne M. in: *Liver and Aging* (K. Kitani, Ed.), p. 3. Elsevier Biomedical Press, Amsterdam 1982.

62. Berezin I. V., Klibanov A. M., Samokhin G. P., Goldmacher V. S., Martinek K.: Methods Enzymol. *44*, 558 (1976).
63. Berezin I. V., Klibanov A. M., Samokhin G. P., Goldmacher V. S., Martinek K. in: *Biomedical Applications of Immobilized Enzymes* (T. M. S. Chang, Ed.), p. 237. Plenum, New-York 1977.
64. Berezin I. V., Klibanov A. M., Martinek K.: Biochim. Biophys. Acta *364*, 193 (1974).
65. Laskin A. I., Mosbach K., Thomas D., Wingard L. B. jr. (Eds): *Enzyme Engineering*, Vol. 8 (Ann. N.Y. Acad. Sci., Vol. 501). New York Acad. Sci., New York 1987.
66. Laane C., Tramper J., Lilly M. D. (Eds): *Biocatalysis in Organic Media.* Elsevier, Amsterdam 1987.
67. Klibanov A. M., Cambou B.: Methods Enzymol. *136*, 117 (1987).
68. Lilly M. D., Harbron S., Narendranathan T. J.: Methods Enzymol. *136*, 138 (1987).
69. Carrea G., Cremonesi P.: Methods Enzymol. *136*, 150 (1987).
70. Luisi P. L., Steinemann-Hofmann B.: Methods Enzymol. *136*, 188 (1987).
71. Laane C., Hilhorst R., Veeger C.: Methods Enzymol. *136*, 216 (1987).
72. Martinek K., Berezin I. V., Khmelnitsky Yu. L., Klyachko N. L., Levashov A. V.: Biocatalysis *1*, 9 (1987).
73. Khmelnitsky Yu. L., Levashov A. V., Klyachko N. L., Martinek K.: Enzyme Microb. Technol. *10*, 710 (1988).
74. Kurganov B. I., Shkarina T. N., Malakhova E. A., Davydov D. R., Chebotareva N. A.: Biochimie *71*, 573 (1989).