Biochimica et Biophysica Acta, 981 (1989) 161–172
Elsevier

BBA 74435

The second E.C. Slater Lecture

Micellar enzymology: its relation to membranology *

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(Received 16 January 1989)

Key words: Micellar enzymology; Membrane; Enzyme; Reversed micellar system

Micellar enzymology, a new trend in molecular biology, studies catalysis by enzymes entrapped in hydrated reversed micelles composed of surfactants (phospholipids, detergents) in organic solvents. The key research problems of micellar enzymology and its relation to enzyme membranology are discussed.

Introduction

Micellar enzymology is a novel physicochemical line of approaches to problems of molecular biology [1–3].

What gave rise to this new discipline? First, we all know that, in the past, the development of molecular enzymology has come about mainly through studies on *free* enzymes. In other words, the highly important experiments designed to elucidate the structures of catalytic centers and physicochemical mechanisms governing biocatalysis could be performed to satisfaction only with enzymes isolated from the living cells in very pure form. Naturally, we must ask whether the properties of enzymes observed 'in vitro' in such 'pure' experiments adequately reflect the conditions under which these enzymes are functioning 'in vivo'. Such doubts are quite equitable in view of the findings that the subcellular structure and the compartmentalization of enzymes play key roles in the regulation of metabolism [4–7]. To use other words, in living cells, enzymes mostly act either *on* or *near to* the 'water/organic medium' interface (that is, on the surface of biological membranes or inside them, or in mobile complexes with macromolecular components of the cell, e.g., with proteins or polysaccharides [8–10]. We are thus facing a contradictory situation: we try to investigate the 'in vivo' action of enzymes in our 'in vitro' studies which usually are carried out simply in water (in aqueous buffer) [11–14].

Second, it is well known also that the main structural pattern of biological membranes is the flat bilayer of lipid molecules. However, the notion of the lipid bilayer as the only possible way of organization of membrane lipids, which represents the essence of the widely accepted fluid mosaic model [15] of biological membranes, does not agree with established facts of structural rearrangements of lipids, for example, from the bilayer to the hexagonal phase (see, for example, Ref. 16 and references therein). Further investigations of the structure of lipid membranes (mainly by the Dutch group; for recent reviews, see Refs. 17–19) resulted in the discovery of other types of non-bilayer lipid structure, in particular, so-called lipidic particles, representing 'reversed micelles' sandwiched between monolayers of the lipid bilayer. The concept of non-bilayer structures in lipid membranes made a basis for a 'metamorphic mosaic' model of biomembranes [20] which explains elegantly many processes occurring in the living cell, such as fusion and compartmentalization of membranes, exo- and endocytosis, lipid flip-flop, etc.

From the point of view of our discussion, the ability of certain proteins to induce the formation of non-bilayer structures upon incorporation into model and biological membranes is of particular significance. Evidence of this kind has been obtained for cytochrome *c* [19], methemoglobin [21], cytochrome *P*-450 [22], proteins of the erythrocyte membrane [23], and hydrophobic polypeptides [24], the latter being regarded as a model of anchoring fragments of ntegral membrane proteins. It is quite possible tha the formation of non-bilayer structures represents a general mechanism

* An abridged version of the second E.C. Slater lecture, delivered at the 14th International Congress of Biochemistry, held in Prague (Czechoslovakia), July 9–16, 1988.

Abbreviation: Aerosol OT (or AOT), sodium bis(2-ethylhexyl) sulfosuccinate.

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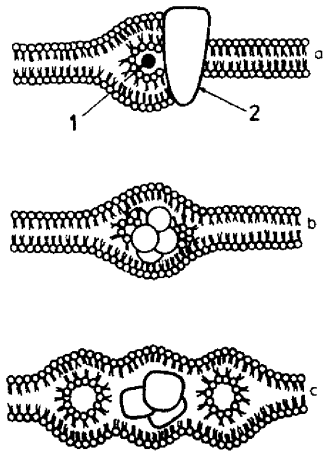

Fig. 1. Assumed non-bilayer structures of the reversed micelle type and their interaction with enzymes (proteins) in biomembranes. (a) Cytochrome *c* (1) and cytochrome *c* oxidase (2) in mitochondria [17]; (b) the enzyme complex of cytochrome *P*-450 in microsomes [22]; (c) integral proteins of photosynthetic membranes [26].

for the protein incorporation into membranes [25–27] and for the regulation of their activity. This assumption is supported by the fact that ATPase [28] and mannosyl transferase [29] show maximal catalytic activity exactly under conditions when the formation of intramembraneous lipidic particles occurs. The same is true also in the case of cell lipases [30,31], for which non-bilayer lipids have been shown to be the best substrates. Fig. 1 illustrates some significant examples.

Taken together, the above data allow one to conclude that model studies of enzymatic catalysis in microheterogeneous media, like the systems of hydrated reversed micelles of surfactants [1–3], are of great importance in understanding of the enzyme functioning in natural lipid systems. In such studies, the free enzymes are dissolved not in aqueous buffers but in colloidal (reversed micellar) solutions of water in organic solvents. By now, many reversed micellar systems containing dozens of different enzymes have been studied; for reviews, see Refs. 32–46. Here, some important new developments in the field of micellar enzymology will be discussed.

General remarks

How are the reversed micelles formed?

Three-component systems composed of organic solvent plus water plus surfactant have been studied in colloidal chemistry for a long time. Fig. 2 shows a typical phase diagram [47]. Depending on the concentration of the individual components, the products formed are: ordinary micelles, lamellar or tubular structures, and finally, the reversed micelles, i.e., associates with a spherical or ellipsoidal shape.

Reversed micellization occurs in completely different solvents, such as hydrocarbons (for example, octane or benzene), long-chain alkanols, chloroform, diethyl ether and in mixtures of these solvents. Widely used detergents, for example, poly(ethylene glycol) derivatives of the Brij, Tween of Triton series, or natural phospholipids, are acceptable micelle-forming material: for review, see the reference list in Refs. 3, 32–46.

The most important properties of the reversed surfactant micelles in organic solvents

Of considerable importance is the fact that reversed micelles exhibit a relatively ordered structure, characterized by a definite (although average) diameter (less than a few nanometers), molecular weight (aggregation number), and packing density.

The dynamics of formation of reversed micelles, their structure, such as their dimensions and the aggregation number as well as the properties of the micromedium, have been under investigation in many laboratories throughout the world. The results of these investigations show that, for instance, the micelles can exchange surfactant molecules with one another and with the organic continuum at a high rate. Moreover, surfactant molecules in the reversed micelle show a continuous vibration which resembles "a feather floating in a stiff wind". Despite the high mobility of surfactant molecules, the micellar interface is rather well defined and impermeable for the organic solvent surrounding the micelle.

Because of their polar core, reversed micelles can solubilize in apolar solvents a large volume of water, up to some tens of molecules per one molecule of the surfactant. The water in the cavity of the hydrated reversed micelle differs from bulk water in its physi-

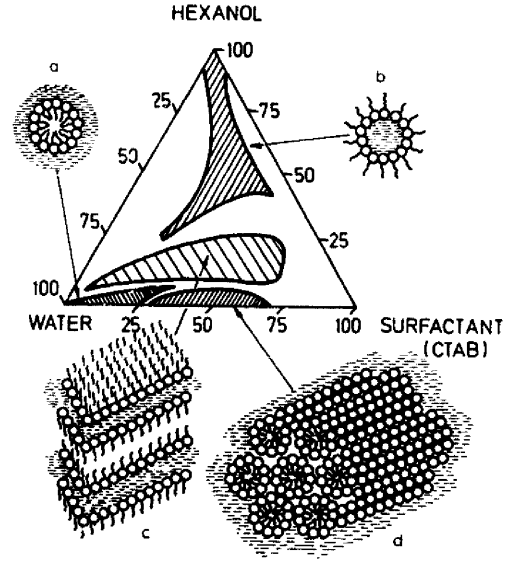

Fig. 2. Phase diagram of the system cetyltrimethylammonium bromide/water/hexanol. (a) Normal micelle; (b) reversed micelle; (c,d) intermediate (lamellar and cylindrical) mesophases. (From Ref. 47).

cochemical properties, i.e., in acidity, microviscosity, polarity, dielectric constant and so forth. For instance, at a low degree of hydration the viscosity of water solubilized in hydrocarbon is 200-times higher than that of bulk water, and its polarity corresponds to the polarity of chloroform (for review, see Ref. 3).

Furthermore, the rate of chemical reactions involving water molecules often increases sharply when reversed micelles are used instead of aqueous solution. For example, the nucleophilic activity of the water solubilized by AOT micelles is at least 10^6-times higher than that of the bulk water [48,49]. However, with the increasing volume of water in the system and with the swelling of the reversed micelles, the difference in the properties of micellar and bulk water becomes less pronounced.

The three following properties of micellar systems are of key importance for enzymology

First, these systems are optically transparent. Second, reversed micelles are formed spontaneously and an equilibrium state is achieved before long: in seconds or minutes, sometimes even earlier. And, third, the dimensions of reversed micelles are easily variable (Fig. 3). In order to vary the dimensions of reversed micelles, it is sufficient to alter the concentration of either water or surfactant in the organic solvent. The higher the quantity of water in the system (horizontal line) the larger is the radius of the micellar particle. On the contrary, as the surfactant concentration increases, the micelles become smaller (vertical line). And finally, if the concentration ratio for both components remains constant, the micelles do not change in structure but increase in number (slanting line).

How can we entrap the enzyme in reversed micelles?

The inclusion of the protein in reversed micelles occurs spontaneously. All you have to do is to add a

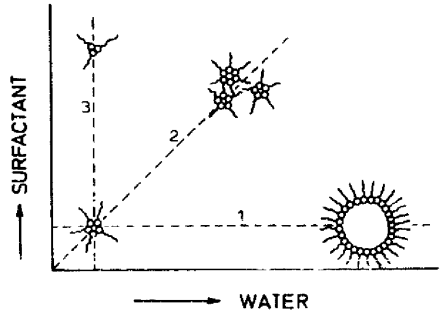

Fig. 3. Effect of the content of water and surfactant on a reversed micellar system. (1) Surfactant concentration is constant, $[H_2O]$ increases (the hydration degree increases): micellar dimensions increase, the number of micelles decreases; (2) [surfactant] and $[H_2O]$ increases in equal proportion (the hydration degree is constant): micellar dimensions remain constant, the number of micelles increases; (3) [surfactant] increases, $[H_2O]$ is constant (the hydration degree decreases): micellar dimensions decreases, the number of micelles increases.

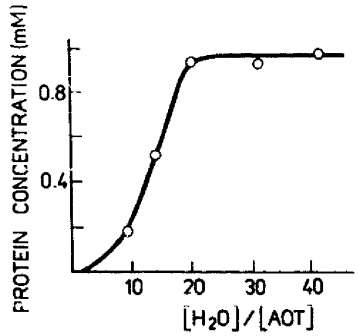

Fig. 4. Dependence of a maximal concentration of the protein (chymotrypsin) solubilized in the system 0.1 M AOT/octane on the hydration degree of the surfactant. (From Ref. 52).

lyophilized enzyme preparation [50] or to inject a stock protein solution [1] into a three-component system containing organic solvent plus surfactant plus water. Shaking or mixing will help to establish the equilibrium (for review, see Ref. 51).

Attention should be drawn to the fact that, for very low values of the ratio $[H_2O]$/[surfactant] (ratio < 1–2), proteins are almost insoluble in the reversed micellar systems. The solubilization of the protein begins only at a certain value of $[H_2O]$/[surfactant] beyond which an increase in the degree of hydration of the surfactant is accompanied by an almost linear increase in the solubility of the protein up to concentrations comparable to those attainable in aqueous solutions. Fig. 4 shows [52] that the solubilization of one molecule of a protein under investigation (chymotrypsin) requires about 1000 molecules of water. The upper break of the curve enables us to estimate the solubility limit of the protein in the system considered, that is to determine the minimal amount of detergent necessary to solubilize one protein molecule. From the data shown in Fig. 4 it was possible to conclude that the solubilization of one chymotrypsin molecule requires about 100 molecules of detergent, that is approximately the amount necessary to cover the whole surface of the protein globule with a monomolecular layer. In principle, large amounts of proteins (about 20 mg/ml) can be solubilized in organic solvents; this is more than enough for physicochemical research.

Structure of protein-containing reversed micelles

The process of the enzyme solubilization in reversed micellar systems results in the formation of hydrated protein-containing reversed micelles, no matter which of the above-listed solubilization procedures has been used; the systems are thermodynamically reversible. As a result, enzyme molecules are located (entrapped) in the inner water cavities of these micelles (Fig. 5). A *hydrophilic* enzyme (E_1) is located in the water core of the micelle and is surrounded by a water layer and a surfactant shell which protect the enzyme against the inactivation by the bulk organic phase. The molecule

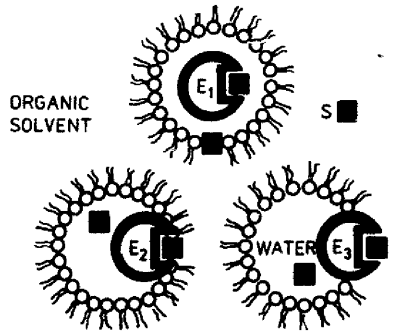

Fig. 5. Schematic representation of the interaction of substrate (or other reagent) molecules (S) distributed in the reversed micellar system with entrapped hydrophilic (E_1), surface-active (E_2), and hydrophobic (E_3) enzymes. (Derived from Refs. 1 and 2).

(E_2) of a *surface-active* enzyme (for example, of lipase) can interact with the interface layer. Finally, molecules of *hydrophobic* proteins (E_3) can in principle come into contact with the phase of the organic solvent.

Another (more complicated) example is given by integral hydrophobic proteins, bacteriorhodopsin [53] and porin [54]. These proteins, when solubilized in reversed micellar solution, induce the formation of dumb-bell-like micellar aggregates in which polar parts of protein molecules are incorporated into 'local' reversed micelles, whereas the central hydrophobic part of the protein freely contacts with the organic solvent. This type of protein-containing reversed micellar aggregates is shown in Fig. 6. The spectral characteristics of rhodopsin solubilized in an apolar organic solvent are almost similar to those of the native protein [55]. Hence, micellar systems are really a useful tool for modeling membrane functions in pseudohomogeneous media.

The above views of the protein-containing reversed micelles is accepted by all authors working in the field [56–63]. However, a considerable contraversy has existed as to their *fine* structural organization. Let us consider a hydrophilic enzyme entrapment in more detail.

Two main opposing models of protein-containing reversed micelles are being discussed in the literature. According to the water-shell model (Fig. 7a), suggested by Luisi and co-workers [35,56,58], the entrapments of the protein molecule is *invariably* accompanied by an

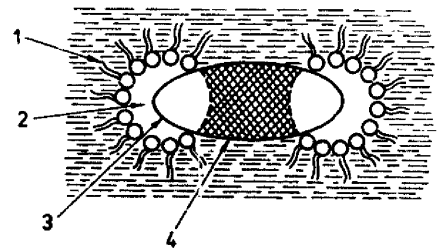

Fig. 6. Schematic representation of the rhodopsin-phospholipid complex in hexane. (1) lipid; (2) water; (3) hydrophilic and (4) hydrophobic fragments of the protein molecule. (From Ref. 55).

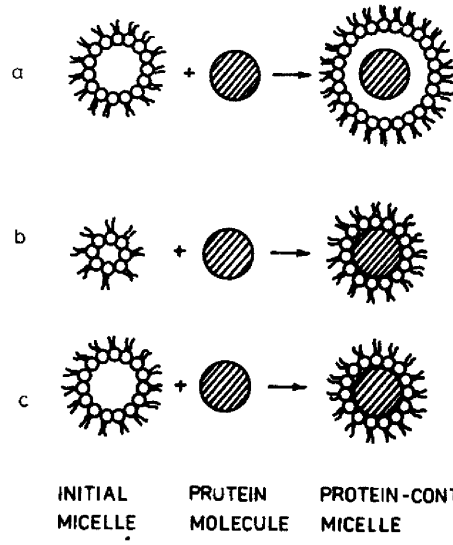

Fig. 7. Models for solubilization of protein molecules (radius r_p) in reversed micelles (outer radius R_m, inner cavity radius r_m) leading to the formation of protein-containing micelles (outer radius R_{mp}, inner cavity radius r_{mp}):
(a) Water-shell model: $R_{mp} > R_m$ and $r_{mp} > r_p$, independent of the r_p/r_m ratio.
(b) Induced-fit model: $R_{mp} > R_m$ and $r_{mp} \approx r_p$, when $r_p > r_m$.
(c) Fixed-size model: $R_m \approx R_{mp}$, when $r_p \leqslant r_m$.

increase in the micelle size and the filled micelle contains more surfactant and water molecules than the initial empty micelle (as the result of redistribution of these micellar components between filled and unfilled micelles). An alternative model, suggested by ourselves [57], assumes that such increase in size occurs only when the inner cavity of the initial empty micelle is smaller than the protein molecule (the induced fit model, see Fig. 7b); in this case the entrapment of the protein can, in principle, result in the increase in the aggregation number as well as the hydration degree of the surfactant. If, on the other hand, the size of the initial water cavity exceeds (or is approximately equal to) that of the protein molecule, then, in contrast to the water-shell model, the protein entrapment may not lead to any substantial increase in the size of the reversed micelle (the fixed-size model, see Fig. 7c).

Ultracentrifugation measurements [57] confirmed the validity of the fixed-size model for a number of water-soluble (hydrophilic) proteins, such as trypsin, chymotrypsin, lysozyme, egg white albumin, horse liver alcohol dehydrogenase and γ-globulin. It was found [57] that the increase in volume, if any, of the reversed micelle as a result of the protein entrapment did not exceed 10% of its initial value, even when the protein molecule and the water cavity of the initial micelle were of the same size. The protein-containing micelle contains practically the same number of both surfactant and water molecules as the unfilled one; in other words, the observed mass of the new aggregate is equal to the sum of masses of the protein and the empty micelle.

Recent experiments with the use of direct physical methods, such as quasi-elastic neutron scattering [59,60] and photon correlation spectroscopy [60] have unambiguously confirmed the validity of the fixed size model. Nevertheless, the model has been subjected to criticism [36,58] on the grounds that it contradicts 'the common sense', because it implies the 'disappearance' of water from the micelle upon the entrapment of the enzyme molecule without any appreciable loss in mass. In order to resolve this discrepancy, we have undertaken ^{13}C-NMR investigations [64] aimed at the elucidation of structural rearrangements occurring in the micelle upon the insertion of the protein molecule, which could explain the surprising phenomenon of the apparent disappearance of water.

As a model system, we used α-chymotrypsin dissolved in the reversed micellar solution of Aerosol OT in octane, because it is this system that has been shown to be best described by the fixed size model by several methods [57,59,60]. The dimensions of the α-chymotrypsin molecule [65] are $40 \times 40 \times 50$ Å. It may be approximated by a sphere of the same volume (about 41 000 Å^3) with a radius of 21.5 Å. The size of inner cavities of reversed AOT micelles in octane depends [66] on the hydration degree of the surfactant expressed as $w = [H_2O]/[AOT]$. In our experiments we used $w = 12$ when the water cavity of the reversed micelle has a radius of 22.1 Å [66]. This means that the volume of the inner cavity of the initial empty micelle (45 200 Å^3) slightly exceeded that of the enzyme molecule, i.e., the conditions required for the realization of the fixed-size model (Fig. 7c) were satisfied. According to this model [57], the amount of water and the aggregation number of AOT are the same both in protein-containing and protein-free micelles. Simple calculations show that in this case water molecules in the protein-containing micelle should form a layer of about 6 Å thickness around the entrapped enzyme (at the above indicated volume ratio of the water cavity to the α-chymotrypsin molecule). In order to keep overall dimensions of the protein-containing micelle unchanged, this water layer should be expelled from the inner cavity of the micelle, which is now almost completely occupied by the enzyme, and penetrate into the surfactant shell towards the organic phase. The situation is depicted schematically in Fig. 8.

^{13}C-NMR measurements indeed confirmed [64] the validity of the above conclusions. It was found that the addition of α-chymotrypsin into the solution of AOT in octane at $w = 12$ caused dramatic changes in the spin-lattice relaxation time (T_1) for carbon atoms situated inside the range of 5–7 Å from the inner micellar interface (between the dotted lines in Fig. 8), whereas T_1 values for carbons lying further along AOT aliphatic chains remained essentially unchanged. The observed shifts in T_1 values were ascribed [64] to the change in

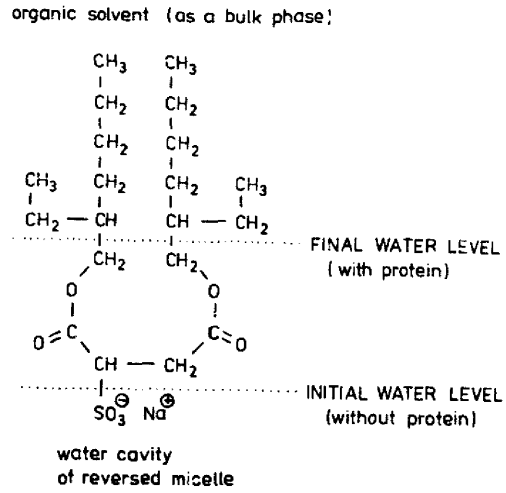

Fig. 8. Scheme of the expulsion of water from the inner cavity of a reversed AOT micelle on entrapment of a protein molecule (the fixed-size model in Fig. 7).

the microenvironment of corresponding carbon atoms by their immersion into the expelled water layer. Quite remarkably, the thickness of the expelled water layer determined from the NMR measurements (5–7 Å) excellently coincides with the value predicted by the fixed-size model (about 6 Å). Inspection of molecular models, constructed on the basis of available data on the conformation of AOT molecules in reversed micelles [67–70], confirms that the suggested expulsion of water from the micellar core is indeed possible: in the areas where AOT polar heads are bonded to the hydrocarbon tails (Fig. 8) there are cavities quite capable of hosting the expelled water molecules.

Our description of the structural rearrangement of water and surfactant molecules in AOT reversed micelles is in excellent agreement with recent results [71] of studies on the hydration of lipid bilayers in biological membranes. It has been shown [71] that under certain conditions the apparent partial molar volume of hydration water in biological membranes can reach zero due to the inclusion of water molecules into large voids in the head-group region of the lipid bilayer, which means, in fact, apparent 'disappearance' of water exactly in the same way as in our model micellar system (in the presence of protein).

Catalytic activity of enzymes in reversed micelles *

It is not surprising that lipolytic enzymes retain their enzymatic function in such microheterogeneous media [73,74] since the presence of an interface is a condition

* Recently catalysis by enzymes entrapped into hydrated surfactant aggregates having lamellar or cylindrical (hexagonal) structure in organic solvents (Fig. 2), has been studied [72] as well.

'sine qua non' for their functioning. The enzymes studied in traditional 'in-water' enzymology are, however, a completely different matter. That is why the first studies [1] on such enzymes carried out in 1977, that is the studies on the catalytic activity of peroxidase and of chymotrypsin entrapped in hydrated reversed micelles prepared from a synthetic detergent in liquid hydrocarbon, stimulated extensive research in this field. As of now there are more than three dozens enzymes whose catalytic behavior in such systems has been studied (for review, see Refs. 3, 32–46 and 51). The kinetics of chemical reactions catalyzed by enzymes entrapped in reversed micelles of amphiphilic compounds in organic solvents obeys, as a rule, the classical Michaelis-Menten equation [75]. On the other hand, enzymes in such a microheterogeneous micellar medium often show completely different catalytic properties compared to those observed in aqueous solutions. Let us consider significant examples.

Substrate specificity

We have studied [76] the specificity of alcohol dehydrogenase solubilized in octane (Fig. 9). The enzyme catalyzes the oxidation of alkanols to the corresponding aldehydes. Fig. 9 shows the dependence of catalytic activity on the length of the substrate molecule. In water, octanol is the best substrate (see the right-hand curve). In colloidal solutions butanol is oxidized faster than other substrates (see the left-hand curve). The phenomenon observed can be simply explained by assuming that the more hydrophobic substrates are extracted to a higher degree with the organic solvent and

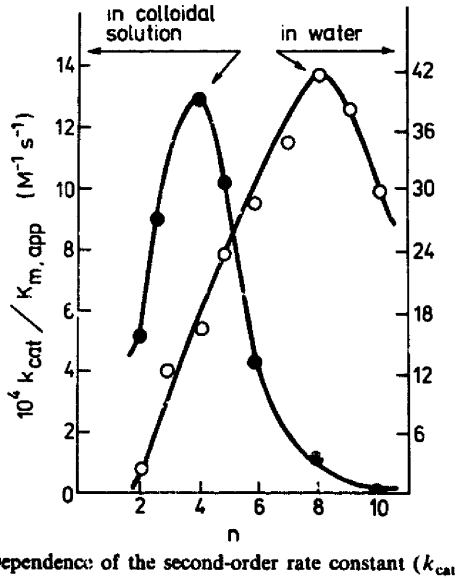

Fig. 9. Dependence of the second-order rate constant ($k_{cat}/K_{m,app}$) for oxidation of aliphatic alcohols, catalyzed by alcohol dehydrogenase from horse liver, on the length of the hydrocarbon fragment (n) in a molecule of substrate, $H(CH_2)_nOH$: ●, the system 0.1 M AOT/octane/water at $[H_2O]/[AOT] = 49$; ○, aqueous solution. Temperature 25 °C, pH 8.8. (From Ref. 76).

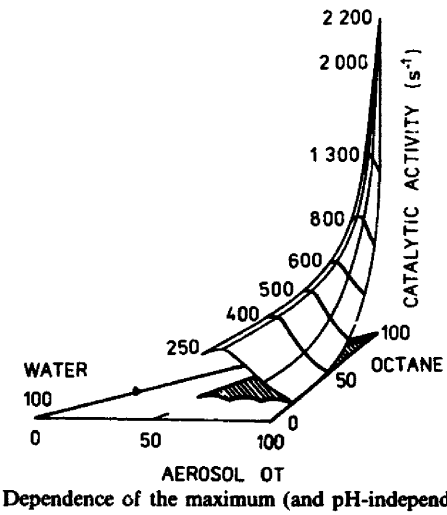

Fig. 10. Dependence of the maximum (and pH-independent) rate of pyrocatechol oxidation, catalysed by laccase entrapped in reversed micelles, on composition of the ternary system AOT-octane-aqueous buffer. In the aqueous solution, the value of k_{cat} is equal to 28 s^{-1}. (From Ref. 77).

their local concentration near the active center is therefore lower.

Superactivity

Another micellar effect, one of the most striking ones, is the increased activity (or superactivity) of the enzymes entrapped. As a criterion of the 'true' catalytic activity we chose a 'pH-independent' value of k_{cat} which is not influenced by trivial effects such as the pH shift and a possible increase in substrate concentration in the micelles. As seen in Fig. 10, the superactivity manifests itself in ternary systems consisting of surfactant, water and organic solvent and having a certain optimum composition [77,78]. With laccase shown in Fig. 10, the increase is about 100-fold compared to the k_{cat} value determined in aqueous solutions.

Other enzymes possessing similarly high superactivity in the micellar medium, are acid phosphatase [79] and peroxidase [80,81].

To understand the mechanism inducing superactivity let us focus our attention on two findings. First, the dependence of the catalytic activity on the molar ratio of water to surfactant concentration is usually represented by a bell-shaped curve, i.e., there is an optimum value of the hydration degree, $w = [H_2O]/[\text{surfactant}]$, for which the catalytic activity of the solubilized enzyme is maximum. The actual value of the optimum hydration degree depends on the nature of the enzyme and the surfactant. Several characteristic examples are given in Ref. 51 (see Fig. 11). No doubt, the optimum is the result of action of numerous factors, which either compensate or supplement each other. One fact only, however, is relevant to our present discussion: under optimum conditions, that is, when the hydration degree is optimum, the inner diameter of the empty micelle prac-

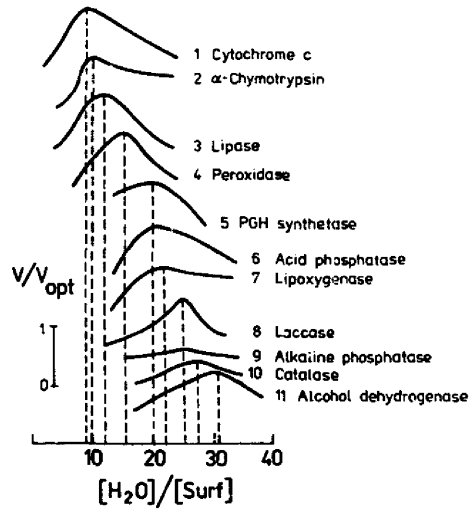

Fig. 11. Regulation of the relative catalytic activity (V/V_{opt}) of different enzymes solubilized in AOT reversed micelles, by variation of the surfactant hydration degree. (From Ref. 51).

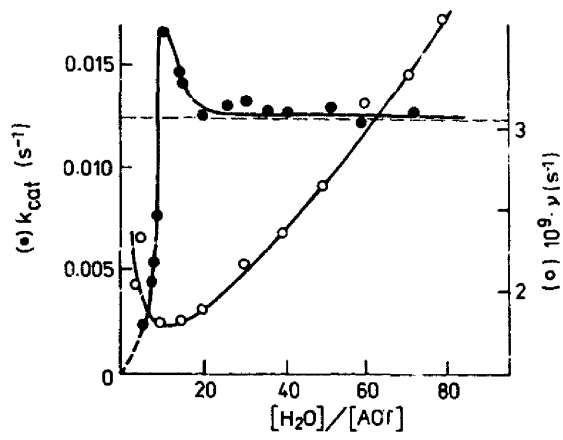

Fig. 13. Dependence of the first-order rate constant (k_{cat}) for deacylation of *N-trans*-cinnamoyl-α-chymotrypsin (●) and rotation frequency (○) for spin-labeled α-chymotrypsin on the H_2O/AOT molar ratio in the system AOT (0.1 M)/octane/water (pH 8.0) at 25 °C. The broken line shows the value of k_{cat} in aqueous solution. (From Ref. 83).

tically corresponds to the size of the molecule of the enzyme entrapped. This conclusion [82–85] is illustrated by Fig. 12, which shows the coincidence of the radii of the different protein molecules (r_p) and corresponding inner cavities of the 'optimum micelles' (r_m).

The second very important finding has come about through studying [83] the signal of an ESR spin label introduced into the active center of solubilized chymotrypsin. As can be seen in Fig. 13, the value of minimal rotation speed of the spin label in the active center of the enzyme (see lower curve) coincides with the optimum of its catalytic activity (see upper curve).

These two results seem to support the following hypothesis. The superactivity is due to the relatively high rigidity of the surfactant shell surrounding the molecule of the solubilized enzyme. The surfactant shell may function as a buffer (or absorber) of excessive fluctuations that usually [11,12] destroy the catalytic conformation in water.

This conclusion has received supporting evidence from studying the catalytic activity of the solubilized enzyme as a function of microviscosity of the core of the micelle [86]. The microviscosity inside the micelle was continuously varied: instead of water (an aqueous buffer) concentrated aqueous solutions of glycerol, various diols and water-soluble organic solvents (such as, for instance, dimethyl sulfoxide) were injected into the micelle. As can be seen in Fig. 14 the catalytic activity of the enzyme increased with increasing microviscosity, regardless of which organic component was used to increase the microviscosity around the enzyme. When a critical value of the latter had been reached the catalytic

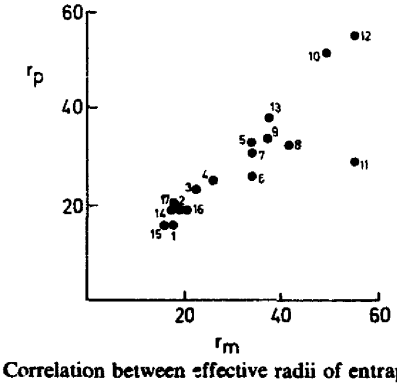

Fig. 12. Correlation between effective radii of entrapped enzyme (r_p) and corresponding optimum aqueous micellar cavities (r_m). Point numbers 1–11, the same as in Fig. 11; 12, alcohol dehydrogenase (r_p = the longest semiaxis of the enzyme molecules); 13, lactate dehydrogenase; 14, trypsin; 15, lysozyme; 16, ribonuclease; 17, pepsin. (From Ref. 51).

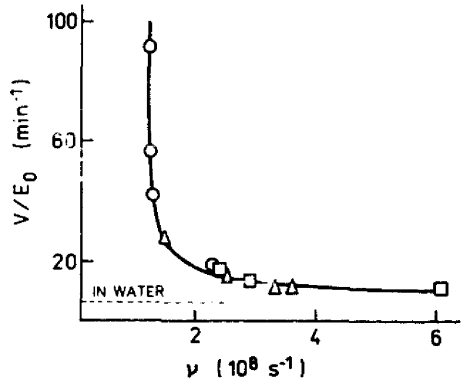

Fig. 14. Relation between the catalytic constant ($k_{cat} = V/E_0$) of α-chymotrypsin catalyzed hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide, and the conformational mobility of the enzyme, expressed in terms of the rotational frequency (ν) of the spin label in the active site of the enzyme. The experiments are performed in the system AOT/water/octane with water-soluble organic components being injected into the reversed micelles: □, dimethyl sulfoxide; △, 2,4-butanediol; ○, glycerol. (From Refs. 51,86).

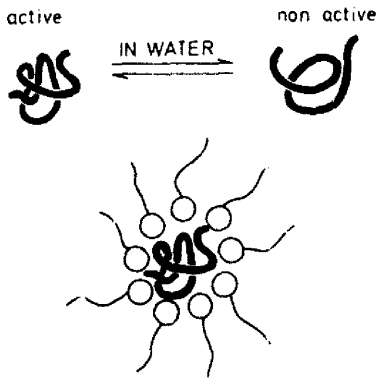

Fig. 15. Scheme of the elimination of spontaneous fluctuations of protein structure inside the reversed micelle, the fluctuations which usually disturb the catalytic conformation in water.

activity of the enzyme rose to a value 100-times higher than in water. This finding (Fig. 14), similarly to those mentioned before (Fig. 13), also shows that the mechanism inducing superactivity in the reversed micelles is the elimination of spontaneous fluctuations of protein structure, fluctuations which usually [11,12] disturb the catalytic conformation of the active center in water (Fig. 15).

A test for the membrane activity of enzymes

A change in the concentration of surfactant in reversed micellar systems carried out at a fixed hydration degree value (w) results in the alteration of the concentration of identical micelles without influencing their size and other properties (see Fig. 3 and accompanying discussion). Hence, the catalytic activity of solubilized enzymes should not depend on the surfactant concentration, if the value of w is kept constant. The validity of this conclusion has been demonstrated for a number of enzymes, such as α-chymotrypsin [75,87], trypsin [75], alkaline phosphatase [72] and lipoxygenase [88] (see Fig. 15, right column). However, there is a group of enzymes, such as peroxidase [81], acid phosphatase [89], laccase [77], and prostaglandin synthetase [90], whose activity strongly depends on the surfactant concentration. The change in catalytic activity can be as high as one or two orders of magnitude, as illustrated by Fig. 16 (left column). All enzymes from this group, in contrast to enzymes from the first group (right column), are characterized by the presence in their molecules of 'anchoring groups of different nature' [90,91]. We suggest that it is the presence of such groups capable of interacting with micellar membrane, that causes the dependence of catalytic activity on the surfactant concentration. The validity of this suggestion was confirmed experimentally [92] in comparative studies of catalytic behavior of α-chymotrypsin in the native state and covalently modified with stearoyl residues. When solubilized in AOT reversed micelles in octane, the native α-chymotrypsin, being a member of the first group of enzymes, did not show any dependence of catalytic activity on the surfactant concentration at a fixed value of w. On the other hand, in the case of the hydrophobized α-chymotrypsin a profound dependence of catalytic activity on the surfactant concentration was observed. These results are shown in Fig. 16 on the panel corresponding to α-chymotrypsin. Thus, by introducing a hydrophobic anchoring group it is possible to convert artificially an enzyme from the first group into an enzyme belonging to the second group.

An important corollary of these findings (Fig. 16) is that the occurrence of the dependence of catalytic activity of an enzyme dissolved in a reversed micellar system on the surfactant concentration can serve as a convenient test for the ability of the enzyme to interact with micellar (and probably also with biological) membranes.

A new strategy for studying oligomeric enzymes

A *theoretical* consideration of the regulation of enzyme activity in systems containing reversibly adsorbing enzymes was recently presented [93]. However, an

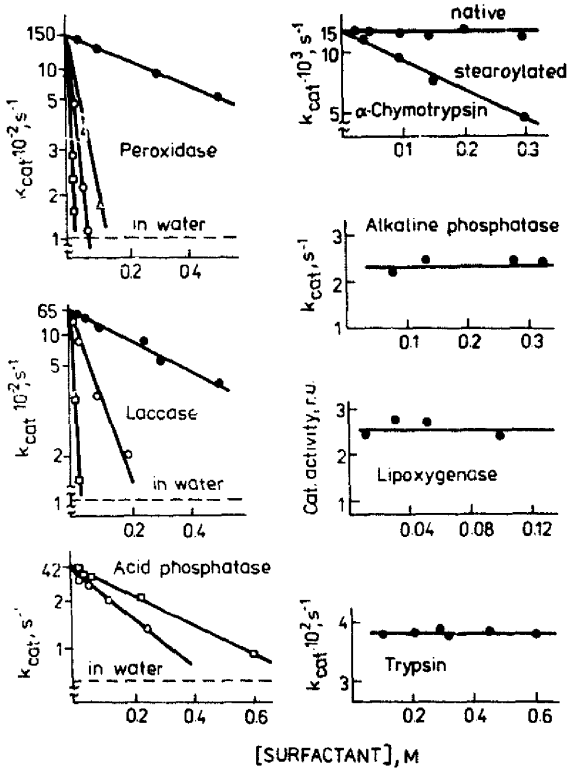

Fig. 16. Regulation of catalytic activity of solubilized enzymes by variation of the surfactant concentration at a constant w value in systems: ●, AOT/water/octane; △, dodecylammonium propionate/water/diethyl ether/benzene; ×, Brij 95/water/cyclohexane; □, lecithin/water/methanol/penthanol/octane. Dashed lines show levels of corresponding catalytic activities in aqueous solution. (From Ref. 51).

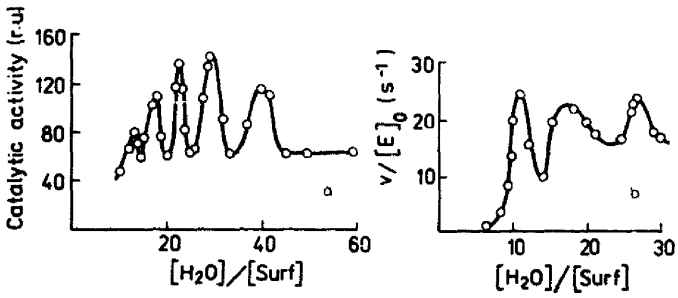

Fig. 17. Regulation of catalytic activity of oligomeric enzymes solubilized in AOT reversed micelles in octane by variation of the surfactant hydration degree: (a) lactate dehydrogenase [95] and (b) γ-glutamyltransferase [96,97].

experimental study of these phenomena is limited by the absence of reliable approaches permitting control of the composition of protein complexes (in order to induce artificially either their assembly or their degradation). Thus, in the case of oligomeric enzymes, the disaggregation of their subunits in vitro is often observed only under the denaturing conditions, which considerably hampers the study of the catalytic properties of the separate subunits [93,94].

These structure–function problems can be solved using systems of hydrated reversed micelles of surfactants in organic solvents as media for the enzymatic reactions under study. The possibility of the activity regulation of an oligomeric enzyme (the composition of its subunit structure being changed in the system of the reversed micelles) was demonstrated in lactate dehydrogenase [95] and γ-glutamyltransferase [96,97].

The dependence of catalytic activity of oligomeric enzymes on $w = [H_2O]/[\text{surfactant}]$ reveals several maxima, as shown in Fig. 17 for the enzymes solubilized in AOT reversed micelles in octane. The molecule of lactate dehydrogenase is composed of four identical subunits (total molecular mass 140 kDa) and represents [98] an ellipsoid with dimensions 74 × 74 × 84 Å. Following the above formulated concept of the geometric fit of the enzyme molecule to the aqueous cavity of reversed micelles under conditions of the optimum catalytic activity (Fig. 12), and using the data [98] for the dimensions of differently packed subunits of lactate dehydrogenase, one may conclude that the maxima at $w = 14$, 18, 22, 31, and 40 on the profile of catalytic activity in Fig. 17a correspond, respectively, to the monomer, dimer, trimer and/or tetramer, and octamer of subunits of lactate dehydrogenase. Following the same line of reasoning, the maxima on the catalytic activity profile for γ-glutamyltransferase (Fig. 17b), which is composed of two subunits with molecular mass 20 and 53 kDa [99,100] were assigned to the light ($w = 11$) and heavy ($w = 17$) subunits, and to their dimer ($w = 26$). Ultracentrifugation experiments [96,97] confirmed the presence in both systems of subunit aggregates predicted on the basis of the catalytic activity studies (Fig 17).

These findings imply that the reversed micelles of surfactants in organic solvents function as *the matrices of adjustable size* permitting regulation of the supramolecular structure and the catalytic activity of oligomeric enzymes.

Applied enzymology

Applied aspects of micellar enzymology are discussed in recent reviews [3,37–46,51]. Applications in fine organic syntheses, in clinical and chemical analyses, in bioconversion of energy and mass, and in medicine, as well as probable future trends in biotechnology, have been exhaustively listed by us before [40]. Here only one impressive example, having a relation to membranology, will be stressed.

Hydrophobized proteins translocating across lipid membranes

As known, micellar media provide a possibility to modify or to convert water-insoluble compounds [3]. In particular, there is a possibility of modification of proteins by water-insoluble reagents [101]. Most recent data reveal the process of protein hydrophobization by phospholipids [102,103] and fatty acids [104] as being quite widespread in living organisms and evidently playing an important role in protein transport [102,103,105]. Hence, it is reasonable to expect [101] that a molecule of a water-soluble protein incapable of passing across the membrane barrier may be converted into a membrane-penetrating form if equipped with a hydrophobic anchor. The key experimental problem of introducing a small number of hydrophobic anchor groups into a protein molecule, was recently solved [101] in our laboratory.

Proteins can be made hydrophobic, for example, by acylation with stearoyl chloride. However, it is very difficult to carry out this reaction in water. As shown in Fig. 18a, the reaction proceeds namely on the surface of microdrops and hence, there is one principal difficulty: the reaction cannot be stopped when the protein is substituted to a low degree. When the protein is cova-

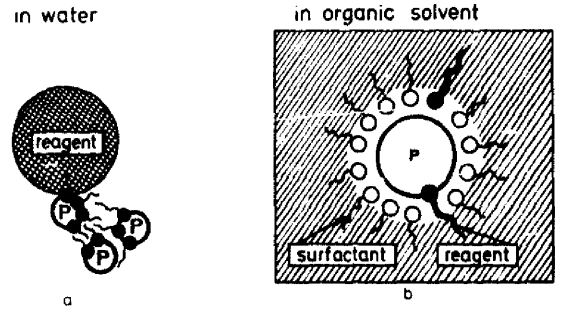

Fig. 18. Protein (P) modification with a water-soluble reagent: (a) in water and (b) in micellar system.

lently hydrophobized in a colloidal solution of water in an organic solvent (Fig. 18b) the situation is completely different [101]. In this case the molecules of the reagent are located around the protein (black bars) and they are diluted with a chemically inert surfactant (white bars). As a result, the protein can be modified even at one site only. In other words, the micellar medium enables us to prevent the protein from substitution to a higher degree.

Conclusive evidences were produced [106,107] on the hydrophobized enzyme to pass through the model membranes (e.g., into multilamellar liposomes). The question remained to be answered whether the translocation of hydrophobized proteins as revealed in the model experiments [106,107] is equally valid for complex biological systems. To this end Kabanov et al. [108] investigated the effect of the ricin A chain on intact B-cells ('Namalva' line). The ricin molecule, a toxin capable of penetrating into the cell and blocking enzyme synthesis in 60 S ribosomes, consists [109] of two polypeptide subunits, A and B-chains. The B-chain is responsible for binding of the toxin on the cell membrane and for translocating the active A-chain into the cell. The toxin A fragment alone (in the absence of a B-chain) possesses weak non-specific toxicity only: it cannot pass through a cell membrane and reach the ribosomes. This 'defect' of the A-chain can be rectified by hydrophobic anchor groups introduced into it; we found [108]: "the toxic effect of the A-chain acylated by stearic acid on B-cells to be close to that of the native ricin". In these experiments the effect of ricin on the cells was measured on the criterion of cell destruction as well as inhibition both of protein (^{35}S-methionine inclusion) and DNA (^{3}H-thymidine inclusion) biosyntheses (Fig. 19).

The mechanism of protein translocation through lipid membrane is not yet fully understood. However, literature data [17–20,110] permit us to assume that artificially hydrophobized proteins can induce the formation of other than bilayer structure in the membrane, such as intermembranous lipidic particles similar to reversed micelles or the hexagonal H_{II} phase; the enzymes are

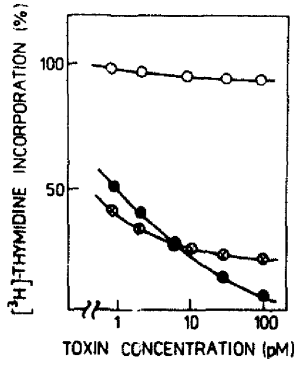

Fig. 19. The action of ○ non-modified and ⊗ stearic acid-acylated ricin A-chains, and ● native toxin on intact β-lymphoma cells ('Namalva' line). (From Ref. 108).

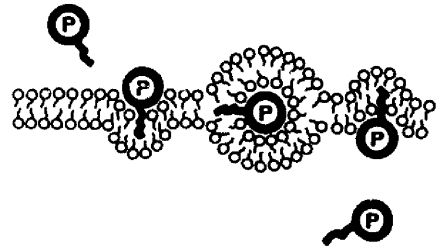

Fig. 20. Hypothetical mechanism of translocation of an artificially hydrophobized protein (P) through the lipid membrane. (From Ref. 108).

included into their inner polar cavity (Fig. 5). These intramembranous lipidic particles have a dynamic character, so that after being formed on one side of the lipid bilayer they can, in principle, dissociate on its opposite side. This means that they may be regarded as possible transmembrane carriers (Fig. 20).

As we understand it the translocation effect of artificially hydrophobized proteins is not only of theoretical, but also of practical importance as it can be utilized for the design of essentially new drugs capable of penetrating into the target cells. For instance, the above suggested [108] principle of imparting transmembrane properties to water-soluble proteins made it possible to realize 'in vivo' the effective transport of fatty acid-acylated antibodies across hematoencephalic barrier into brain [111].

Conclusion

By the present time, more than three dozen enzymes have been studied in reversed micellar systems due to efforts of about 20 laboratories all over the world [3]. Evaluating the rapid development of micellar enzymology, it is to be noted that a simple idea of placing the enzyme molecule into a microreactor formed by the reversed micelle has led to consequences that a decade ago, when studies in this field started [1], were difficult to predict. In fact, together with new fundamental approaches to modeling the enzyme-membrane organization (Fig. 1) and to the mechanistic study of enzymatic catalysis (see Figs. 11–14 and accompanying discussion), micellar enzymology has also given seminal results opening new prospects in practical chemistry, fine organic syntheses, clinical and chemical analyses, bioconversion of energy and other applied areas [40].

In our opinion, such outstanding progress is the result of the two following main advantages of reversed micellar systems. First, these microheterogeneous systems provide means for easy dissolution of both hydrophilic and hydrophobic substances under standard conditions, thus giving extremely wide possibilities to vary the nature of molecular objects used in research. In other words, the molecule of the solubilized enzyme (depending on its nature) can come into contact, in

principle, with water-soluble, with surface-active and also with organophilic (water-insoluble) substrates (Fig. 5). The micellar solution of water in organic solvents (stabilized by amphiphilic compounds) thus represents a universal, that is, all-purpose, microheterogeneous medium suitable for enzymatic reactions [2].

Second, reversed micelles ensure a strict *nano*compartmentalization of solubilized macromolecules, thus permitting a controlled build-up of subunit structures (see, for example, Fig. 17 and accompanying discussion). Other impressive examples are the synthesis of conjugates of natural and synthetic macromolecules of a *predetermined* composition [112], and homogeneous enzyme immunoassay in reversed micelles [113]. The sensitivity of the assay procedure can be optimized by adjusting the size of the reversed micelles (i.e., the surfactant hydration degree).

The achievements of micellar enzymology over the past several years, both in the fundamental and applied aspects, give us good reason to expect rapid progress in this field [114].

References

1 Martinek, K., Levashov, A.V., Klyachko, N.L. and Berezin, I.V. (1977) Dokl. Akad. Nauk SSSR (Russ.) 236, 920–923; (1978) Engl. Edn. 236, 951–953.
2 Martinek, K., Levashov, A.V., Khmelnitski, Yu.L., Klyachko, N.L. and Berezin, I.V. (1982) Science (Wash. DC) 218, 889–891.
3 Martinek, K., Levashov, A.V., Klyachko, N.L., Khmelnitski, Yu.L. and Berezin, I.V. (1986) Eur. J. Biochem. 155, 453–468.
4 Green, D., Murer, E. and Hultin, H.O. (1965) Arch. Biochem. Biophys. 112, 635–647.
5 Kun, E. (1972) in Biochemical Regulatory Mechanisms in Eucaryotic Cells (Kun, E. and Grisolia, S., eds.), pp. 271–303, Academic Press, New York.
6 Ottaway, J.H. and Mowbray, J. (1977) Curr. Top. Cell. Regul. 12, 107–208.
7 Welch, G.R. (1977) Progr. Biophys. Mol. Biol. 32, 103–191.
8 Masters, C.J. (1981) CRC Crit. Rev. Biochem. 11, 105–143.
9 Werner, M., Garrett, C., Chin, A. and Klempner, M. (1982) Clin. Chem. 28, 2351–2358.
10 Wombacher, H. (1983) Mol. Cell. Biochem. 56, 155–164.
11 Fersht, A. (1977) Enzyme Structure and Mechanism, Freeman, San Francisco.
12 Berezin, I.V. and Martinek, K. (1977) Physicochemical Fundamentals of Enzymatic Catalysis (Russ.), Higher-School Press, Moscow.
13 Dixon, M. and Webb, E.C. (1979) Enzymes, 3rd Edn., Longman, London.
14 Bender, M.L., Koniyama, M. and Bergeron, R. (1982) Bioorganic Chemistry of Enzymatic Catalysis, Wiley, New York.
15 Singer, S.J. and Nicholson, G.L. (1972) Science 175, 720–731.
16 Borovjagin, V.L., Vergara, J.A. and McIntosh, T.J. (1982) J. Membr. Biol. 69, 199–212.
17 De Kruijff, B., Cullis, P.R., Verkleij, A.J., Hope M.J., Van Echteld, C.J.A. and Taraschi, T.F. (1985) in The Enzymes of Biological Membranes (Martonosi, A.N., ed.) 2nd Edn., Vol. 1: Membrane Structure and Dynamics, pp. 131–204, Plenum Press, New York.
18 Cullis, P.R., Hope, M.J. and Tilcock, C.P.S. (1986) Chem. Phys. Lipids 40, 127–144.
19 Cullis, P.R., De Kruijff, B., Hope, M.J., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C.P.S., Madden, T.D. and Bally, B. (1983) in Membrane Fluidity in Biology (Aloia, R.C., ed.), pp. 39–81, Academic Press, New York.
20 Cullis, P.R., De Kruijff, B., Hope, M.J., Nayar, R. and Schmid, S.L. (1980) Can. J. Biochem. 58, 1091–1100.
21 Chupin, V.V., Ushakova, I.P., Bondarenko, S.V., Vasilenko, I.A., Serebrennikova, G.A., Evstigneeva, R.P., Rozenberg, G.Ya. and Koltsova, G.N. (1982) Bioorgan. Khim. (Russ.) 8, 1275–1280.
22 Stier, A., Finch, S.A.E. and Börsterling, B. (1978) FEBS Lett. 91, 109–112.
23 Hah, J.S., Hui, S.W. and Jung, C.Y. (1983) Biochemistry 22, 4763–4769.
24 Zvonkova, E.N., Khabarova, E.I., Vasilenko, I.A. and Evstigneeva, R.P. (1984) Bioorgan. Khim. (Russ.) 10, 1401–1408.
25 Van Echteld, C.J.A., De Kruijff, B., Verkleij, A.J., Leunissen-Bijvelt, J. and De Gier, J. (1982) Biochim. Biophys. Acta 692, 126–138.
26 Murphy, D.J. (1982) FEBS Lett. 150, 19–26.
27 Stier, A., Finch, S.A.E., Greinert, R. and Hohne, M. (1982) in Liver and Aging (Kitani, K., ed.), pp. 3–14, Elsevier Biomedical Press, Amsterdam.
28 Hui, S.W., Stewart, T.P., Yeagle, P.K. and Albert, A.D. (1981) Arch. Biochem. Biophys. 207, 227–240.
29 Jensen, J.W. and Schitzbach, J.S. (1984) Biochemistry 23, 1115–1119.
30 Dawson, R.M.C. (1982) J. Am. Oil Chem. Soc. 59, 401–406.
31 Dawson, R.M.C., Irvine, R.F., Brag, J. and Quinn, P.J. (1984) Biochem. Biophys. Res. Commun. 125, 836–842.
32 Khmelnitsky, Yu.L., Levashov, A.V., Klyachko, N.L. and Martinek, K. (1984) Russ. Chem. Rev. 53, 319–331.
33 Levashov, A.V., Klyachko, N.L., Khmelnitski, Yu.L. and Martinek, K. (1984) in Surfactants in Solutions (Mittal, K.L., ed.), Vol. 2, pp. 1069–1091, Plenum Press, New York.
34 Luisi, P.L. and Straub, B. (eds.) (1984) Reverse Micelles, Plenum Press, New York.
35 Luisi, P.L. (1985) Angew. Chem. Int. Engl. Edn. 24, 439–450.
36 Luisi, P.L. and Magid, L.J. (1986) CRC Crit. Rev. Biochem. 20, 409–474.
37 Luisi, P.L. and Laane, C. (1986) Trends Biotechnol. 4, 153–159.
38 Waks, M. (1986) Proteins (Struct. Function Genet.) 1, 4–15.
39 Shield, J.W., Ferguson, H.D., Bommarius, A.S. and Hatton, T.A. (1986) Ind. Eng. Chem. Fundam. 25, 603–612.
40 Martinek, K., Berezin, I.V., Khmelnitsky, Yu.L., Klyachko, N.L. and Levashov, A.V. (1987) Coll. Czech. Chem. Commun. 52, 2589–2602.
41 Luisi, P.L. and Steinmann-Hofmann, B. (1987) Meth. Enzymol. 136, 188–216.
42 Laane, C., Hilhorst, R. and Veeger, C. (1987) Meth. Enzymol. 136, 216–229.
43 Martinek, K., Berezin, I.V., Khmelnitsky, Yu.L., Klyachko, N.L. and Levashov, A.V. (1987) Biocatalysis 1, 9–15.
44 Levashov, A.V., Klyachko, N.L., Khmelnitsky, Yu.L. and Martinek, K. (1987) Ann. N.Y. Acad. Sci. 501, 205–209.
45 Khmelnitsky, Yu.L., Levashov, A.V., Klyachko, N.L. and Martinek, K. (1988) Enzyme Microb. Technol. 10, 710–724.
46 Khmelnitsky, Yu.L., Levashov, A.V., Klyachko, N.L. and Martinek, K. (1988) Biotekhnologiya (Russ.) 4, 292–309.
47 Ahmad, S.I. and Friberg, S. (1972) J. Am. Chem. Soc. 94, 5196–5199.
48 Sunnamoto, J., Kondo, H. and Akimaru, K. (1978) Chem. Lett. 821; J. Org. Chem. 43, 3584–3589.
49 Martinek, K., Levashov, A.V., Pantin, V.I. and Berezin, I.V. (1978) Dokl. Akad. Nauk SSSR 238, 626–629.
50 Menger, F. and Yamada, K. (1979) J. Am. Chem. Soc. 101, 6731–6734.
51 Khmelnitsky, Yu.L., Kabanov, A.V., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) in Structure and Reactivity in

Reverse Micelles (Pileni, M.P. and Troyanowsky, C., eds.), Elsevier, Amsterdam, in press.
52 Klyachko, N.L. (1983) Ph.D. Thesis, Moscow State University, Moscow.
53 Montal, M. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E., eds.), pp. 221–229, Plenum Press, New York.
54 Wirz, J. and Rosenbuch, J.P. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E., eds.), pp. 231–238, Plenum Press, New York.
55 Ramakrishnan, V., Darszon, A. and Montal, M. (1983) J. Biol. Chem. 258, 4857–4860.
56 Bonner, E.J., Wolf, R. and Luisi, P.L. (1980) J. Solid-Phase Biochem. 5, 255–268.
57 Levashov, A.V., Khmelnitsky, Yu.L., Klyachko, N.L., Chernyak, V.Ya. and Martinek, K. (1982) J. Colloid Interface Sci. 88, 444–57.
58 Zampieri, G.G., Jaeckle, H. and Luisi, P.L. (1986) J. Phys. Chem. 90, 1849–1853.
59 Fletcher, P.D.I., Howe, A.M., Perrins, N.M., Robinson, B.H., Toprakcioglu, C. and Dore, J.C. (1984) in Surfactants in Solution (Mittal, K.L. and Lindman, B., eds.), pp. 1745–1758, Plenum Press, New York.
60 Fletcher, P.D.I., Robinson, B.H. and Tabony, J. (1986) J. Chem. Soc., Faraday Trans. I 82, 2311–2321.
61 Petit, C., Brochette, P. and Pileni, M.P. (1986) J. Phys. Chem. 90, 6517–6521.
62 Shen, E., Goklen, K.E., Hatton, T.A. and Chen, S.H. (1986) Biotechnol. Progr. 2, 175–186.
63 Chatenay, D., Urbach, W., Nicot, C., Vacher, M. and Waks, M. (1987) J. Phys. Chem. 91, 2198–2201.
64 Shapiro, Yu.E., Budanov, N.A., Levashov, A.V., Klyachko, N.L., Khmelnitsky, Yu.L. and Martinek, K. (1989) Coll. Czech. Chem. Commun. 54, 1126–1134.
65 Birktoft, J.J., Blow, D.M., Henderson, R. and Steitz, T.A. (1970) Phil. Trans. Royal Soc. B257, 67–80.
66 Eicke, H.F. and Rehak, J. (1976) Helv. Chim. Acta 59, 2883–2891.
67 Ueno, M., Kishimoto, H. and Kyogoku, Y. (1978) J. Colloid Interface Sci. 63, 113–119.
68 Martin, C.A. and Magid, L.J. (1981) J. Phys. Chem. 85, 3938–3944.
69 Maitra, A.N. and Eicke, H.F. (1981) J. Phys. Chem. 85, 2687–2691.
70 Maitra, A. (1984) J. Phys. Chem. 88, 5122–5125.
71 Scherer, J.R. (1987) Proc. Natl. Acad. Sci. USA 84, 7938–7942.
72 Klyachko, N.L., Levashov, A.V., Pstezhetski, A.V., Bogdanova, N.G., Berezin, I.V. and Martinek, K. (1986) Eur. J. Biochem. 161, 149–154.
73 Hanahan, D.J. (1952) J. Biol. Chem. 195, 199–206.
74 Misiorowski, R.L. and Wells, M.A. (1974) Biochemistry 13, 4921–4927.
75 Martinek, K., Levashov, A.V., Klyachko, N.L. and Berezin, I.V. (1981) Biochim. Biophys. Acta 657, 277–294.
76 Martinek, K., Khmelnitsky, Yu.L., Levashov, A.V. and Berezin, I.V. (1982) Dokl. Akad. Nauk SSSR (Russ.) 263, 737–741.
77 Pshezhetsky, A.V., Klyachko, N.L., Pepaniyan, G.S., Mercker, S. and Martinek, K. (1988) Biokhimiya (Russ.) 53, 1013–1016.
78 Pshezhetsky, A.V., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) Biocatalysis, submitted.
79 Levashov, A.V., Klyachko, N.L., Pshezhetski, A.V., Berezin, I.V., Kotrikadze, N.G., Lomsadze, B.A. and Martinek, K. (1986) Dokl. Akad. Nauk SSSR (Russ.) 289, 1271–1274.
80 Martinek, K., Klyachko, N.L., Levashov, A.V. and Berezin, I.V. (1983) Dokl. Akad. Nauk SSSR (Russ.) 269, 1491–1493; Engl. Edn. 269, 69–71.
81 Klyachko, N.L., Levashov, A.V. and Martinek, K. (1984) Mol. Biol. (Russ.) 18, 1014–1031; Engl. Edn. 18, 830–833.
82 Levashov, A.V., Klyachko, N.L. and Martinek, K. (1981) Bioorgan. Khim. (Russ.) 7, 670–679.
83 Likhtenstein, G.I., Belonogova, O.V., Levashov, A.V., Klyachko, N.L., Khmelnitsky, Yu.L. and Martinek, K. (1983) Biokhimiya (Russ.) 48, 379–386.
84 Martinek, K., Klyachko, N.L., Levashov, A.V. and Berezin, I.V. (1983) Dokl. Akad. Nauk SSSR (Russ.) 269, 491–493.
85 Kurganov, B.L., Tsetlin, L.G., Malakhova, E.A., Lankin, V.Z., Levashov, A.V. and Martinek, K. (1985) J. Biochem. Biophys. Methods 11, 177–184.
86 Klyachko, N.L., Bogdanova, N.G. and Levashov, A.V. (1988) unpublished results.
87 Fletcher, P.D.I., Rees, G.D., Robinson, B.H. and Freedman, R.B. (1985) Biochim. Biophys. Acta 832, 204–214.
88 Klyachko, N.L. and Schevchuk, T.V. (1988) unpublished results.
89 Mevkh, A.T., Sudina, G.F., Lagutina, I.O. and Levashov, A.V. (1985) Biokhimiya (Russ.) 50, 1719–1723.
90 Welinder, K.G. and Smillie, L.B. (1972) Can. J. Biochem. 50, 63–90.
91 Reinhammer, B. (1979) in Copper Proteins and Copper Enzymes (Brown, B., ed.), pp. 1–35, Academic Press, New York.
92 Kabanov, A.V., Levashov, A.V. and Martinek, K. (1986) Vestnik MGU, Ser. 2 - Khimiya (Russ.) 27, 591–594.
93 Kurganov, B.I. and Loboda, N.I. (1979) J. Theor. Biol. 111, 707–723.
94 Muronetz, V.I. and Nagradova, N.K. (1984) Immobilized Oligomeric Enzymes (Russ.), Nauka Publ., Moscow.
95 Klyachko, N.L., Mercker, S., Vakula, S.V., Levashov, A.V. and Martinek, K. (1988) Dokl. Akad. Nauk SSSR (Russ.) 298, 1479–1481.
96 Kabanov, A.V., Nametkin, S.N., Evtushenko, G.N., Chernov, N.N., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) Biochim. Biophys. Acta, 996, in press.
97 Namyotkin, S.N., Kabanov, A.V., Evtushenko, G.N., Chernov, N.N., Berezov, T.T., Klyachko, N.L., Martinek, K. and Levashov, A.V. (1989) Bioorg. Khim. (Russ.), 15, No. 1, in press.
98 Squire, P.G. and Himmel, M.E. (1979) Arch. Biochem. Biophys. 196, 165–177.
99 Tate, S.S. and Meister, A. (1981) Mol. Cell. Biochem. 39, 357–368.
100 Loginov, V.A., Chernov, N.N. and Berezov, T.T. (1980) Bull. Exp. Biol. (Russ.) 7, 58–60.
101 Levashov, A.V., Kabanov, A.V., Berezin, I.V. and Martinek, K. (1984) Dokl. Akad. Nauk SSSR (Russ.) 278, 246–248; (1985) Engl. Edn. 278, 295–297.
102 Rothman, S.S. (1980) Am. J. Physiol. 238, G. 391–G 402.
103 Nesmeyanova, M.A. (1982) Mol. Biol. (Russ.) 16, 821–829.
104 Schmidt, M.F.G. (1983) Curr. Top. Microbiolog. Immunol. 102, 101–121.
105 Zilberstein, A., Snider, M.D., Porter, M. and Lodish, H.F. (1980) Cell 21, 417–427.
106 Levashov, A.V., Kabanov, A.V., Nametkin, S.N., Martinek, K. and Berezin, I.V. (1985) Dokl. Akad. Nauk SSSR (Russ.) 284, 306–309.
107 Kabanov, A.V., Nametkin, S.N., Levashov, A.V. and Martinek, K. (1986) Biol. Membr. (Russ.) 22, 985–995.
108 Kabanov, A.V., Levashov, A.V., Alakhov, V.Y., Kravtsova, T.N. and Martinek, K. (1989) Coll. Czech. Chem. Commun. 54, No. 3, in press.
109 Sjur, O. (1987) Nature 328, 474–475.
110 De Kruijff, B. (1987) Nature 329, 587–588.
111 Chekhonin, V.P., Morozov, G.V., Kashparov, I.A. and Ryabukhin, I.A. (1988) Biotekhnologiya (Russ.) 4, 648–651.
112 Kabanov, A.V., Alakhov, V.Y., Klinskii, E.Y., Khrutskaya, M.M., Severin, E.S., Levashov, A.V. and Kabanov, V.A. (1988) Dokl. Akad. Nauk SSSR (Russ.), 302, 735–738.
113 Kabanov, A.V., Khrutskaya, M.M., Budavari, M., Klyachko, N.L. and Levashov, A.V. (1988) Dokl. Akad. Nauk SSSR (Russ.), in press.
114 Martinek, K. (1989) Biochem. Int., in press.