MICELLAR ENZYMOLOGY: POTENTIALITIES IN APPLIED AREAS (BIOTECHNOLOGY)

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Micellar enzymology, a new trend in molecular biology, studies the catalysis by enzymes entrapped into hydrated reversed micelles of surfactants (detergents, phospholipids) in organic solvents. The effect of solubilization on enzymatic properties is briefly considered. Applications of such biocatalytic systems in fine organic syntheses, in clinical and chemical analyses, and in medicine, as well as probable future trends in biotechnology are discussed.

1. INTRODUCTION

Biocatalysis was widely adopted for practical use a long time ago[1-4]. Nevertheless, new prospects for biotechnology have been opened by the novel physicochemical trend in molecular biology, micellar enzymology. This discipline studies the catalysis by enzymes solubilized by surfactants (detergents, phospholipids, *etc.*) in organic solvents.

The retention of the enzymatic function in such microheterogeneous media is not surprising for lipolytic enzymes[5,6] since the presence of an interface is an obligatory condition for their functioning. The enzymes of traditional in-water enzymology are quite a different matter. That is why the first reports on the catalytic activity of peroxidase[7] and chymotrypsin[7-10] entrapped into hydrated reversed micelles of a synthetic detergent in liquid hydrocarbons initiated subsequent conducive researches (Fig. 1); to date about 30 enzymes have been involved in the catalytic activity studies as follows from recent reviews[11-16].

Key research problems of micellar enzymology and its relation to enzyme membranology have been recently[16] discussed in detail. Here we shall try to identify and rationalize some general principles and key trends that may prove to be important for applied enzymology (biotechnology).

2. BASIC PROPERTIES OF REVERSED MICELLES

Amphiphilic molecules of surfactants in water/organic solvent media spontaneously form spherical or ellipsoidal associates (micelles). As is well known, there are normal micelles that exist in water (at rather low concentrations of organic additives), and reversed micelles that form in organic solvents (at a moderate water content). Fig. 2 gives an idea of the structure of normal and reversed micelles.

Reversed micellization[17-21] occurs in quite different solvents, such as hydrocarbons (for example, octane or benzene), long chain alkanols, chloroform, diethyl ether, including their mixtures. Widely used detergents (sodium bis(2-ethylhexyl)-sulfosuccinate, *i.e.*, Aerosol OT, poly(ethylene glycol) derivatives of Brij, Tween or

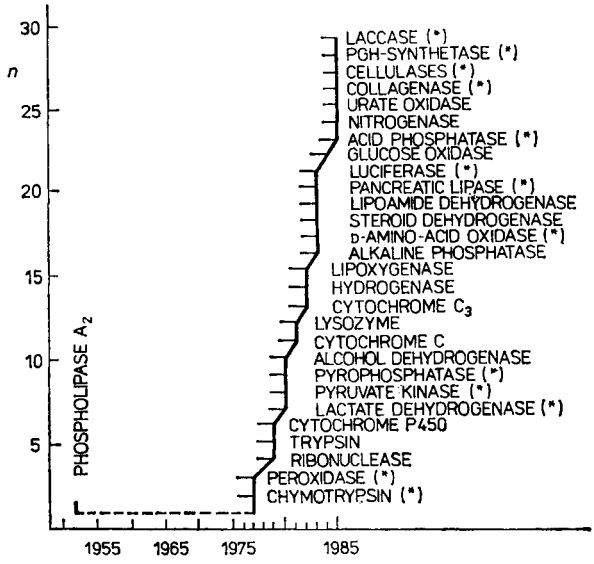

FIG. 1

Time dependence of the number of enzymes (n) studied in reversed micellar systems. Micellar enzymology was conceived in 1977 as follows from the subsequent conducive researches of enzymatic catalysis in reversed micellar media. For particular enzymes, see reviews[11-16]. The catalytic activity of the enzymes marked with an asterisk was studied for the first time in our laboratories

Triton series, *etc.*) or natural phospholipids are acceptable as micelle-forming material. For details, see reference lists in recent reviews[11-16] as well.

Of considerable importance is the fact that reversed micelles exhibit relatively ordered structure (Fig. 2), characterized by a definite (although average) radius (less than a few nanometers), molecular weight (aggregation number), and packing density[17-21]. Due to their polar core, reversed micelles are able to solubilize in apolar solvents large amounts of water (or other polar substances) up to some tens of molecules of the solubilized compound *per* molecule of the surfactant.

Such a capability of the multicomponent system, consisting of water, surfactant(s), and organic solvent(s), seems to be due to a dynamic construction of reversed micelles; they can exchange surfactant molecules with each other and with the organic continuum at a high rate. One of the iportant exchange mechanisms involves a coalescence of the micelles upon collision[22-25]. As a result, the residence time of the surfactant molecule in a micelle is about 10^{-7} s (refs[26,27]). Moreover, surfactant molecules in the reversed micelle show a continuous vibration which resembles "a feather floating in a stiff wind"[28]. Despite the high mobility of surfactant molecules, the micellar interface in many cases is rather well defined[29-31] and impermeable to the organic solvent surrounding the micelle[28,32].

Water in the cavity of the hydrated reversed micelle differs from bulk water by its physico-chemical properties (acidity, microviscosity, polarity, dielectric constant, *etc.*)[17-21]. An important parameter is the molar ratio of water to surfactant. This ratio, more than the absolute amount of water or surfactant present in the organic solvent, determines most of the structural and physical properties of reversed micelles[33,34]. For instance, at low degrees of hydration the viscosity of water solubilized by Aerosol OT in hydrocarbons is 200 times higher than that of bulk water, and its polarity corresponds to that of chloroform[35,36]. Another example is a high chemical activity of solubilized water. Namely, the nucleophilic capacity of the water molecules solubilized by Aerosol OT micelles in octane is at least 10^3 times higher than that of the bulk water[37,38]. However, reversed micelles usually swell, as the amount of water in the system increases[33,34], and therefore the difference in the properties of micellar and bulk water becomes less pronounced. Hence, reversed micelles can be viewed as microreactors of easily variable dimensions containing aqueous microdroplets ("water pools"[22]) whose physical properties can be continuously modulated (through the water content).

3. UNIVERSAL (ALL-PURPOSE) MEDIUM FOR ENZYMATIC REACTIONS

The hydrated reversed micelles of surfactants in organic solvents offer a unique opportunity for dissolving proteins under such standard conditions so that a protein molecule can choose for itself an optimal microenvironment corresponding to its nature (optimal in terms of thermodynamics), see Fig. 3. In fact, a molecule of

a hydrophilic protein (E_1) can avoid direct contact both with the organic solvent and the micellar interface and become localized in the aqueous core of the reversed micelle. At the same time, the surface-active enzymes (lipases) can interact with the surface layer of the reversed micelle, or even be partially buried in it (E_2). Finally, the typical membrane (hydrophobic) enzymes can come into contact even with the organic solvent (E_3).

As to substrate molecules, they may also be partitioned between the aqueous core and the surface layer of a reversed micelle, or the organic continuum, respectively. Hence, the molecule of solubilized enzyme is able (depending on its nature) to come into contact, in principle, both with water-soluble and/or surface-active and/or organophilic substrates.

The micellar solution of water in organic solvents (stabilized with amphiphilic compounds) represents thus a universal (all-purpose)[11] microheterogeneous medium for enzymatic reactions.

4. METHODS

The enzyme-containing micellar systems can be prepared by three different procedures:

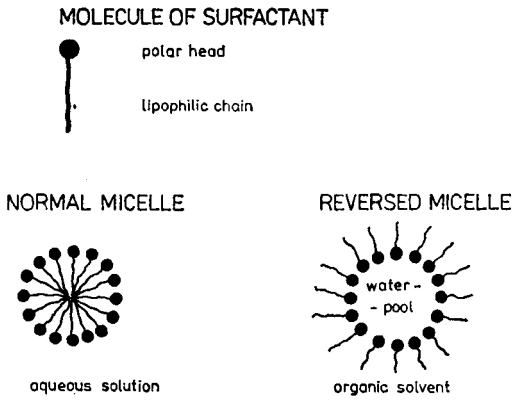

FIG. 2

Schematic representation of normal and reversed micelles (in cross-section). In contrast to normal micelles in aqueous solution, the polar head groups of the surfactant molecules in reversed micelles are directed towards the interior of the aggregate and form a polar core which can solubilize water (the "water pool"); the lipophilic chains are exposed to the solvent

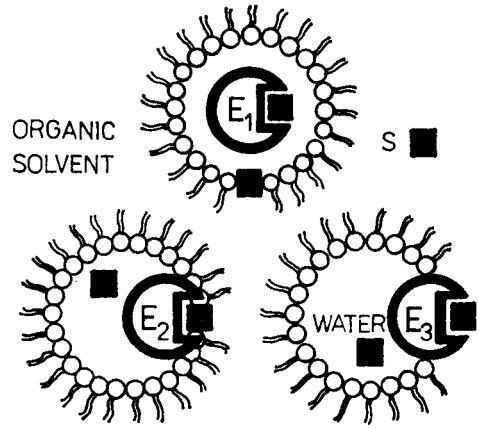

FIG. 3

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[7,11]

(i) by direct injection[7] (the most commonly used method), *i.e.* injecting a small amount of concentrated aqueous stock protein solution into the organic solution of the surfactant. If the right conditions are chosen (concentration of protein and water content of the micellar system), a clear solution is obtained simply by hand-shaking;

(ii) by extraction[8] of a lyophilized preparation (powder) of the protein with a micellar solution containing water;

(iii) by phase transfer[39] in a biphasic system that consists of approximately equal volumes of aqueous protein solution and organic solvent with surfactant; the last method is extensively developed by Luisi and coworkers[40–42].

There are[11] three important characteristics that distinguish the micellar (pseudo-homogeneous) solution from all the enzyme-containing macroheterogeneous systems developed before, such as liquid biphasic media (particularly emulsions), liposomes, suspensions with enzymes bound to the surface or entrapped into supports of various kinds, *etc.*; for review, see ref.[16].

First, the creation of the micellar systems does not call for special techniques as it involves the simple dissolution of an enzyme in a liquid water/organic medium.

Second, the water content and hence the amount of water in the enzyme micro-environment (inside the reversed micelle) can be varied in a wide range, from a practically "dry" solution to a system containing comparable amounts of aqueous and organic components.

Third, the micellar solution of water in organic solvents is optically transparent, which allows the use of conventional spectral methods to observe the structure of solubilized enzymes and the course of enzymatic reactions. The methods applied were listed before[16].

After the enzymatic reaction is completed the solubilized enzyme can be regenerated by the phase transfer method[40] or by precipitation with acetone[43]. Then the product can be isolated by precipitation of the surfactant with acetonitrile[44] or other suitable organic solvent.

5. CATALYTIC PROPERTIES OF SOLUBILIZED ENZYMES

The kinetics of chemical reactions catalyzed by enzymes entrapped into reversed micelles of surfactants in organic solvents obeys, as a rule, the classical Michaelis–Menten equation[7]. However, the kinetic theory[45] of the enzymatic reactions proceeding in such a microheterogeneous medium should take into account a partition of the substrate molecules between the pseudophase of micelles and the bulk phase of organic solvent.

Catalytic properties of solubilized enzymes are exhaustively discussed in recent reviews[11–16]. One of the most striking effects is a superactivity of the entrapped

enzyme (studied in terms of pH-independent values of k_{cat}, which are free of trivial effects of the pH-shift and a possible increase in substrate concentration inside micelles). As a rule, the superactivity reveals itself under an optimal composition of ternary systems composed of surfactant, water, and organic solvent. An example is given in Fig. 4.

The acceleration effect (compared with the k_{cat} value in aqueous solution) is more than 100 for peroxidase[48,49], about 300 for acid phosphatase[50], and 60 for laccase[46]. In our opinion, the superactivity is due to a relatively high rigidity of the surfactant shell surrounding the solubilized enzyme molecule[51], which may function as a damper against superfluous fluctuations that usually destroy the catalytic conformation in water.

6. ANALYTICAL APPLICATIONS

The development of micellar media allows the ideology of well-known enzymatic analytical approaches[1-4,52,53] to be extended to water-insoluble compounds. The

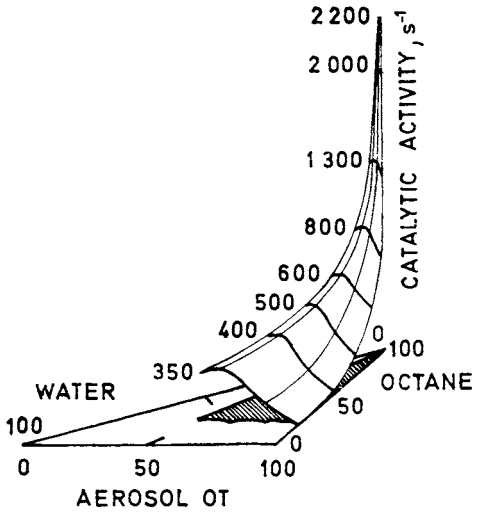

FIG. 4

Dependence of the maximal (and pH-independent) rate of pyrocatechol oxidation, catalyzed by laccase entrapped in reversed micelles, on composition of the ternary system Aerosol OT–octane–aqueous buffer From ref.[46]. For the phase diagram (and determination of the boundaries of reversed micellar phase), see ref.[47]

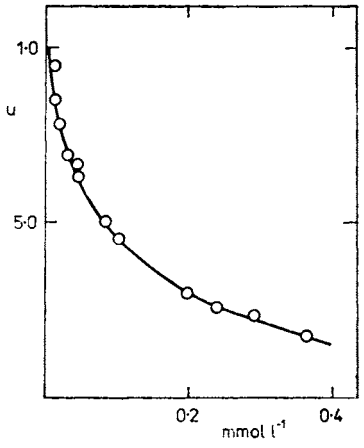

FIG. 5

Inhibition of lipoxygenase by 2′,3′,4′,5′-tetrabenzoyl-1,5-dihydroflavin (mmol l^{-1}) in the system Aerosol OT (0·05M)/ octane / / water (0·05M Tris buffer, pH 8·5). Enzyme activity in relative units (u). From ref.[54]

gist of the matter is that enzymes can interact easily with water-insoluble substrates, inhibitors, activators, *etc.*, in micellar media as is schematically presented in Fig. 3. It gives us a novel horizon for environmental protection, especially for the express analytical assay of pesticides, and other poorly water-soluble compounds, those polluting the environment or even affecting man. For instance, Kurganov *et al.*[54] applied enzymes (lipoxygenase and D-amino-acid oxidase) to testing hydrophobic derivatives of vitamin B_2, see Fig. 5.

The second, but not less important aspect of the micellar approach, stems from the fact that the enzyme entrapped in the reversed micelle often becomes more catalytically active and stable, which results in a higher sensitivity (and reliability) of the analysis. Such situation is encountered in the case of the bioluminescent assay based on firefly luciferase[55]. The enzyme responds to the presence of ATP with luminescence that can be easily registered:

$$\text{ATP} + \text{luciferin} + \text{O}_2 \rightarrow \text{AMP} + \text{pyrophosphate} + \\ + \text{oxyluciferin} + \text{CO}_2 + \text{LIGHT}.$$

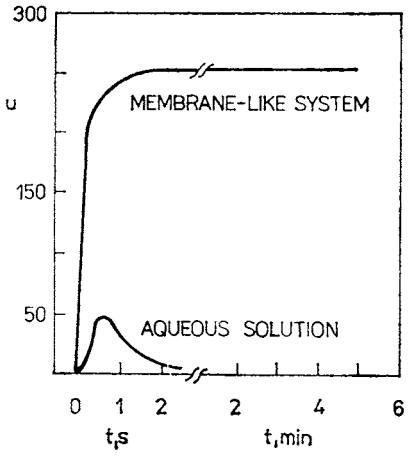

FIG. 6

Kinetic curves of luminescence in relative units (u) in the firefly luciferase-catalyzed reactions: In aqueous solution and in the system Brij 96 (0·01M) + octane + water (9%, v/v). From ref.[55]

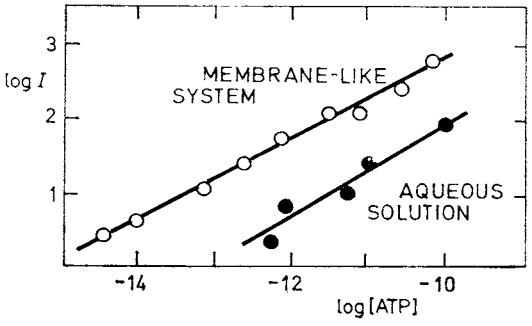

FIG. 7

The bioluminescent assay for ATP determination using firefly luciferase: In aqueous (buffer) solution and in the system 0·25M Brij 96 in cyclohexane + 16% (v/v) of buffer. Ordinate — concentration of ATP, abscissa — intensity of luminiscence. From ref.[56]

TABLE I

Exploitation of reversed micelles in organic solvents as a medium in applied enzymology

Applied areas[a]	Work of priority	Ref.
Fine organic synthesis		
1. General principles	Martinek *et al.*, 1977	7, 62
2. Conversion of water-insoluble compounds:		
a) long chain alkanols by alcohol dehydrogenase	Martinek *et al.*, 1981	64
b) progesterone by steroid dehydrogenase	Hilhorst *et al.*, 1983	57—59
c) glycerides (or related compounds) by lipases	Malakhova *et al.*, 1983	75—78
d) linoleic acid by lipoxygenase	Luisi *et al.*, 1984	79
e) prostanoids by prostaglandin synthetase	Mevkh *et al.*, 1985	80
3. Peptide synthesis catalyzed by chymotrypsin	Lüthi and Luisi, 1984	65
Clinical and chemical analyses		
4. Bioluminescent assay (using firefly luciferase)	Belyaeva *et al.*, 1983	55, 56
5. Luminol-mediated assay for oxidase reactions	Visser and Santema, 1984	97
6. Detection of water-insoluble compounds (using D-amino-acid oxidase and lipoxygenase)	Kurganov *et al.*, 1985	54
7. Enzyme immunoassay (using peroxidase)	Eremin *et al.*, 1986	81
8. Chemiluminescence detection of enzymatically generated hydrogen peroxide	Hoshino and Hinze, 1987	82
Bioconversion of energy and mass		
9. Biophotolysis of water (using hydrogenase)	Hilhorst *et al.*, 1982	83
10. Hydrolysis of cellulose to glucose (catalyzed by cellulases)	Sinitsyn *et al.*, 1983	84

Therapy		
11. Drug carriers in organism,	Speiser, 1976	67
nanogranulated enzymes	Abakumova *et al.*, 1985	68, 69
12. Medicines for outward application (using collagenase and some proteases)	Yarovaya *et al.*, 1986	85
13. Hydrophobized proteins with membrane-activity	Kabanov *et al.*, 1985	66
Protein chemistry		
14. Determination of the molecular weight and effective size of proteins by ultracentrifugation	Levashov *et al.*, 1981	86, 87
15. Covalent modification of proteins by water-insoluble compounds	Levashov *et al.*, 1984	43, 66
16. Trapping of labile intermediates (acylchymotrypsin) in enzymatic catalysis	Levashov *et al.*, 1981	88
17. Selective extraction and separation of proteins[b]	Göklen and Hatton, 1985	73, 74
Miscellaneous		
18. Cryoenzymology	Douzou, 1980	89
19. Separation techniques	Armstrong, 1985	90
20. Cofactor regeneration (both enzymatic and electrochemical)	Hilhorst *et al.*, 1983	57—59

[a] As a medium with a low content of water, liquid two-phase systems of water/water-immiscible organic solvent type, particularly, "water in-oil" emulsions are often used for enzymatic syntheses, as well; for review, see refs[62,91–94]. Another biocatalytic tool is a suspension of enzyme powder in organic solvents, developed for both organic synthesis[14,91,95] and chemical analysis[96]. In contradistinction to the micellar (pseudohomogeneous) medium, all the systems mentioned are macroheterogeneous (opaque, having diffusion limitations and so on). [b] For the development of the method for biomembrane solubilization by means of extraction of proteolipid complexes with organic solvents, see historical essay[16].

The luminescent signal observed in microheterogeneous medium of the surfactant/ /water/organic solvent type becomes much higher compared with that observed in the aqueous solution (Fig. 6), and, as a result, it is possible[56] to detect ATP in a concentration as low as 10^{-15} mol l^{-1}, *i.e.* 10^{-18} mol ATP *per* assay (Fig. 7).

7. ORGANIC SYNTHESES

First, water-insoluble (or poorly soluble) compounds such as steroids, prostanoids, alkaloids, fats, can be subjected to the biocatalytic conversion. For instance, Veeger and coworkers[57-59] succeeded in enzymatic modification of progesterone by molecular hydrogen.

Second, in the traditional medium for enzymatic processes, *i.e.* in water, the equilibrium of many important reactions is shifted to a great extent toward initial reagents. This shift occurs, first of all, in the processes where initial reagents are ionized and, therefore, strongly hydrated, as well as in those where water forms as a product, for example, in the sugar or amino acid condensations, dehydration, *etc.* The unfavourable (in terms of thermodynamics) situation can be improved by conducting the enzymatic reaction in a biphasic water/water-immiscible organic solvent system[60,61]. Here the equilibrium can be shifted (in order to increase the product yield) by lowering the water content in the reaction medium and/or by selecting an organic solvent which can efficiently extract the product.

Colloidal solution of water in organic solvents represents, in fact, a variation of the biphasic liquid system[62,63]. The micellar approach succeeded[64] in changing 10^6-fold the equilibrium constant of the reaction of alcohol oxidation to the corresponding aldehyde (under the action of alcohol dehydrogenase), see Fig. 8. Using the same physico-chemical principle[60-64], peptide synthesis[65] has been recently performed in a micellar medium.

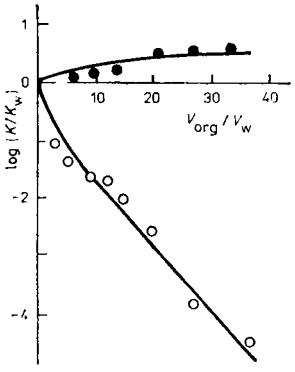

FIG. 8

Dependence of the equilibrium constant, for isobutanol oxidation to isobutyraldehyde, on the volume ratio of organic solvent to water (V_{org}/V_w), using cationic (●) and anionic (○) surfactants. K is equilibrium constant in the mixture organic solvent–water, K_w is equilibrium constant in water. From ref.[64]

8. MEDICAL APPLICATIONS

Proteins entrapped in reversed micelles may be chemically modified by hydrophobic (water-insoluble) reagents (such as stearoyl chloride)[43]. Hydrophobized enzyme preparation can interact with biomembranes, cross them[66] and, therefore, should possess new therapeutic properties.

Reversed surfactant micelles in nonpolar solvents have also been successfully used for the production of new carriers for drug transportation in the organism[67], namely, of polymeric enzyme-containing nanogranules (or nanocapsules), *i.e.* particles of colloidal dimensions (less than a few dozens of nanometers[68,69]).

9. CONCLUSION

Applications of reversed micellar media are not confined to the above examples. Suffice it be to mention many other applied areas[59,70,71], see Table I. Particularly, micellar solution of water in organic solvents is perfectly applicable to solubilization of multienzyme systems[45,57,58] and cells[72] as well. Another impressive example is an efficient method for the continuous recovery of proteins from fermentation and cell-culture media by selective solubilization in reversed micelles[73,74].

Generally speaking, solubilized (or nanogranulated[68]) enzymes in organic solvents might be successfully employed in all fields where aqueous biocatalytic systems have been used so far.

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