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Enzymes in Low Water Systems

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ABSTRACT: Water is fundamental for enzyme action and for formation of the three-dimensional structure of proteins. Hence, it may be assumed that studies on the interplay between water and enzymes can yield insight into enzyme function and formation. This has proven correct, because the numerous studies that have been made on the behavior of water-soluble and membrane enzymes in systems with a low water content (reverse micelles or enzymes suspended in nonpolar organic solvents) have revealed properties of enzymes that are not easily appreciated in aqueous solutions. In the low water systems, it has been possible to probe the relation between solvent and enzyme kinetics, as well as some of the factors that affect enzyme thermostability and catalysis. Furthermore, the studies show that low water environments can be used to stabilize conformers that exhibit unsuspected catalytic properties, as well as intermediates of enzyme function and formation that in aqueous media have relatively short life-times. The structure of enzymes in these unnatural conditions is actively being explored.

KEY WORDS: water, reverse micelles, enzymes and organic solvents, solvent-protein interactions.

I. INTRODUCTION

Because enzymes evolved and work in water, it is obvious that most of our current knowledge of enzymes has derived from experiments in which water was the most abundant component. However, under these conditions it is difficult to study the relation between enzymes and water, that is, the addition of more water to systems with excess water simply results in dilution. In consequence, to explore the interplay between water and enzymes, it became necessary to design novel experimental systems. To this end, several systems, such as protein films at various levels of hydration (Rupley et al., 1983) were developed, but there are now two widely used systems in which the relation between water and enzymes can be experimentally approached. One involves the entrapment of enzymes in reverse mi-

celles (Luisi et al., 1988), and the other the dispersion of enzymes in nonpolar solvents (Klibanov, 1989). These latter systems differ drastically from those that have been used traditionally, and likewise there are differences between the two. Nonetheless, these two nonconventional systems have a common factor, that is, the amount of water in which enzymes are placed is much lower than in the usual biochemical systems.

Here we review experiments with enzymes dispersed in organic solvents or entrapped in reverse micelles. There are several reviews (Luisi et al., 1988; Martinek et al., 1989; Pileni, 1989; Gupta, 1992; Dordick, 1989, 1992; Gómez-Puyou et al., 1992; Westcott and Klibanov, 1994; Nicot and Waks, 1995; Bru et al., 1995) that deal with the characteristics of the systems and the behavior of enzymes in such conditions. As some are rather recent, some overlapping was inevitable. However, we mainly focus on the question of whether in these systems the use of water as a variable parameter has provided further information into enzyme function, structure, and dynamics. Prior to the description on the function and structure of enzymes in these latter nonconventional systems, a brief review of their general characteristics seems appropriate.

II. CHARACTERISTICS OF REVERSE MICELLES

Reverse micelles are spherical water droplets surrounded by a monolayer of closely packed surfactant molecules dispersed in a solvent of low polarity (Luisi et al., 1988). In reverse micelles, the polar heads of the surfactant molecules are in contact and hydrated by water molecules that lie in the interior of the micelle; the hydrophobic tails of the surfactant molecules are oriented and in contact with the surrounding organic solvent. These water droplets are thermodynamically stable due to the presence of the interfacial surfactant layer that prevents unfavorable direct contact between water and the organic solvent.

For the formation of reverse micelles, the surfactants that have been most often used are negatively charged dioctyl sodium sulfosuccinate (AOT) and positively charged cetyltrimethylammonium bromide (CTAB); with the latter, a cosurfactant such as hexanol is also introduced (Hilhorst et al., 1984). It is relevant that phospholipids have also been used for fabrication of reverse micelles (Darszon and Shoshani, 1992; Peng and Luisi, 1990), albeit less is known of this type of micelles.

In studies with enzymes entrapped in reverse micelles, the system has two important characteristics. One is that at a fixed concentrations of surfactant, the water pool inside the micelles varies in proportion to the amount of water that is introduced, that is, as water is increased the dimensions of the water pool increases (Luisi et al., 1988); this relationship holds until the system becomes unstable. Another important feature is that reverse micelles are optically transparent. When enzymes are transferred to the interior of reverse micelles, the system continues to be transparent, and, thus, enzymes entrapped in a space of low water content become amenable for spectroscopic studies (Voss et al., 1987).

At a constant surfactant concentration, the progressive addition of water results in an increase in micellar size and in the ratio of water to surfactant molecules; the latter is generally referred to as Wo. Thus, there is a relationship between Wo and the dimensions of the micelles, and, in fact, a linear relationship between Wo and the radius of the water droplet has been proposed empirically (Pileni et al., 1985; Bru et al., 1995):

$$rw \text{ (in nanometers)} = 0.175\ Wo$$

In the thermodynamically stable region, the dimensions of the micelles may range from 0 to 10 nm (Zulauf and Eicke, 1979).

When water and surfactant concentrations are varied proportionally, the concentration of reverse micelles changes, but their size remains constant (Luisi et al., 1988). At the water and surfactant concentrations generally used, the concentration of reverse micelles in the organic solvent may range from 0.1 to 10 m*M*. As most of the studies are carried out with n*M* or μ*M* concentrations of enzymes, it is envisaged that a single protein molecule is entrapped in one micelle; hence, the number of empty micelles by far exceeds the number of protein-filled micelles. The concentration of substrates that is used in experiments with reverse micelles is generally in μ*M* or m*M*.

With respect to effective substrate concentrations in reverse micelles systems, there has been a long-standing argument about whether the concentration of a water-soluble substrate should be referred to the overall volume of the system or to the volume of the water phase. Some researchers (Verhaert et al., 1992) indicated that the concentration of polar and apolar substrates should be in terms of the total volume of the system. The rationale was that substrates solubilized in aqueous solutions are similar to substrates dispersed by a detergent, and thus the overall volume of the system should be the point of reference. On the other hand, other groups (Martineck et al., 1986) argued that for a water-soluble substrate, the important volume is that of the water phase, that is, the concentration of substrate that the enzyme recognizes. There are several reviews and research articles on this subject, as well as on other factors that contribute to enzyme kinetics in reverse micelles (García Carmona et al., 1992; Ferreira and Gratton, 1992; Martineck et al., 1986: Bru et al., 1989, 1990, 1995; Pérez-Gilabert et al., 1992; Oldfield, 1990; Khmelnitsky et al., 1990; van Erp et al., 1991; Verhaert et al., 1990a, 1990b, 1992).

A. Properties of Water in Reverse Micelles

The properties and state of water in the interior of reverse micelles formed with AOT have been studied with respect to its dielectric constant (Wong et al., 1977) and freezing point (Douzou et al., 1978). It has also been shown by spectroscopic techniques, such as NMR and ESR spin labeling (Haering et al., 1989), and differential scanning calorimetry (Hauser et al., 1989; Luisi et al., 1990) that the differences with bulk water are more marked at Wo below 10. At higher Wo, the properties of micellar water approach those of bulk water. NMR data of micelles formed with phospholipids and CTAB (Darszon and Shoshani, 1992; Kernen et al., 1997) generate a similar picture. From these findings, the conclusion has been reached that the water molecules in closest distance to the polar heads of the surfactant molecules are those that exhibit the largest differences from bulk water. These characteristics arise from the strong interactions of water molecules with the charged polar surface of the micellar wall.

It is pertinent to note that the aforementioned properties of water in reverse micelles have been determined in the absence of entrapped proteins. On the basis of catalytic activity and spectroscopic data, some proteins very likely lie in the water pool of the micelle, that is, the "water-shell" model (Wolf and Luisi, 1981). Nevertheless, one would like to know about the state and the amount of water in which the enzyme is carrying out catalysis. Unfortunately, this problem is difficult to approach.

In regard to the entrapment of proteins in reverse micelles, it is relevant that pro-

teins with dimensions larger than those calculated for reverse micelles at a given Wo can be entrapped in reverse micelles in a catalytically active state. This has led to the notion that proteins build their own micelle (Chatenay et al., 1985). However, knowledge of the state of water with these enzymes in reverse micelles is also lacking. Evidently, information on the state of water in protein-filled micelles is badly needed, because in such an environment enzymes carry out catalytic conversions that often differ from those observed in standard aqueous media.

B. Reverse Micelles Exchange Contents

When enzyme-filled micelles are mixed with substrate-filled micelles, catalysis takes place. This clearly indicates that micelles can transfer their content to other micelles. The events and mechanisms involved in such phenomena have been studied (Fletcher et al., 1987) and critically discussed by Bru et al. (1995). It is visualized that in reverse micellar systems, transformation of substrate into product involves various steps: (1) collision of substrate and enzyme-filled micelles, (2) fusion and formation of a transient micellar dimer, (3) exchange of contents, and (4) division of the dimer with regeneration of two micelles. The exchange rate is of the order of 10^6 to 10^8 M^{-1} s^{-1}. Substrates that are soluble in the organic phase can also be catalytically transformed by enzymes entrapped in the water pool of reverse micelles. In this latter case, it is thought that micellar fusion is not a requisite for enzyme action, that is, substrate can partition from the apolar solvent into the water phase of the micelle through a process that is faster than the rate of micellar fusion and division. With either type of substrate, the rates at which substrates reach the enzyme are substantially faster than the kcat of most enzyme reactions (1 to 100 s^{-1}), and, thus, it can be assumed that for most enzymes, exchange of micellar contents would not be rate limiting for steady-state catalysis (Bru et al., 1995). However, this situation changes with very fast enzymes, such as catalase, which has a kcat of 3.8×10^7 s^{-1}; indeed, Haber et al. (1993) found that under first-order conditions, splitting of H_2O_2 was limited by exchange of substrate between substrate and enzyme-filled micelles.

III. GENERAL FEATURES OF THE SYSTEMS WITH ENZYMES DISPERSED IN ORGANIC SOLVENTS

In a general protocol for measuring the activity of enzymes dispersed in organic solvents (Zaks and Klibanov, 1988a), the starting material is an enzyme dissolved in the desired aqueous buffer, and thereafter lyophilized. The powder is suspended in an organic solvent that has a known amount of water; this is followed by addition of substrate. Incubation is performed under vigorous stirring and at the desired times, aliquots are withdrawn for assay of product. When a water-soluble cofactor is needed, it is added to the enzyme prior to lyophilization. Under these conditions some of the first enzymes studied, that is, alcohol oxidase, polyphenol oxidase, and alcohol dehydrogenase (Zaks and Klibaonov, 1988a) and subtilisin and chymotrypsin (Zaks and Klibanov, 1988) exhibited catalytic activity. Up to now, numerous studies have shown that under these conditions, many other enzymes are capable of carrying out catalysis. However, in the overall results there is a particular point that must be stressed: in all cases, water, albeit

in relatively low amounts, is an essential ingredient for enzyme catalysis.

Because water is an essential component of catalysis by enzymes dispersed in organic solvents, one of the first questions that arises is whether in such conditions the enzyme is surrounded by a shell of water molecules. If this is the case, then the enzyme would not be actually working in organic solvents, instead the enzyme would be working in a water cage. However, a substantial number of reports convincingly demonstrate that enzymes can catalyze with amounts of water that are below monolayer coverage (Zaks and Klibanov, 1988a; Dordick, 1992; Adlercreutz, 1991). Thus, enzymes do work in organic solvents, even though they possesses bound water molecules that are not removed by the organic solvents.

In light of these findings, the question that now arises is if these bound water molecules are essential for catalysis or whether they merely resist removal by organic solvents. In an important work, Zaks and Klibanov (1988a) determined the activity of three unrelated enzymes (alcohol oxidase, polyphenol oxidase, and alcohol dehydrogenase) dispersed in different solvents and different amounts of water. In all solvents activity increased with the amount of water in the system, but significantly they observed that with the more hydrophobic solvents, less water was required for catalysis. The conclusion was that activity depended on the amount of water that was bound to the enzyme, and not on the overall content of water in the system.

Using a highly sensitive radioactive technique, Gorman and Dordick (1992) explored the stability of bound water (T_2O) in chymotrypsin, subtilisin Carlsberg, and horseradish peroxidase in different organic solvents. Their results show that removal of bound T_2O by a given solvent is related to its dielectric, partition coefficient, and capacity to solubilize water. The nature of the enzyme was also a factor in the stability of bound T_2O, but the contribution of the solvent was more important. Thus, the overall data led to the concept that in the low water range, enzymes work better in nonpolar than in apolar solvents because of their lower capacity to remove or solubilize the tightly bound water molecules.

Additional direct evidence for the existence of water molecules that resist removal by organic solvents has come from analysis of the crystal structure of enzymes that have been crystallized in water and thereafter exposed to organic solvents (Fitzpatrick et al., 1993, 1994; Allen et al., 1996; Yennawar et al., 1994, 1995; Schimtke et al., 1997). The data show that indeed there are water molecules in particular regions of the protein that resist removal by organic solvents. Furthermore, regions in which the solvent molecule is in close range of protein residues were also detected.

The picture that emerges from various studies with enzymes dispersed in organic solvents is that for expression of catalysis, enzymes need to have some water molecules. This is in full agreement with the original observations of Rupley et al. (1983; and see Rupley and Careri, 1991) and Finney and Poole (1984) in protein films. Therefore, it would appear that if a few tightly bound enzyme water molecules are preserved, enzymes are indeed capable of carrying out catalytic conversions, independently of which is the predominant component of the system. Indeed, in supercritical fluids with low amounts of water, enzymes can also express good catalytic activity (Hammond et al., 1985; Randolph et al., 1988; Kamat et al., 1993).

In this context, Gorman and Dordick (1992) hypothesized that "it may be feasible to engineer (via site-directed mutagenesis) polar and charged amino acids onto the surface of the protein so that water in the

vicinity of such amino acids would be less likely to partition from the protein into the organic solvent". Engineering of enzymes to work in organic solvents is a field that is actively being pursued (Chen and Arnold, 1991; Chen et al., 1991; Arnold, 1993).

IV. THE EFFECT OF WATER AND SOLVENT ON CATALYSIS BY ENZYMES IN REVERSE MICELLES AND WHEN DISPERSED IN ORGANIC SOLVENTS

A common feature of enzymes in all nonconventional systems is that in the very low range of water concentrations, enzymes exhibit activities that are much lower than in aqueous media; another common feature is that as water is increased, activities increase. However, the activity of enzymes dispersed in organic solvents or entrapped in reverse micelles often respond with a different pattern to increasing water concentrations. The unique features that enzymes exhibit in the two systems will be highlighted, as they provide insight into properties of enzymes that are not easily, or are even impossible to see in aqueous solutions.

A. Catalysis in Reverse Micelles with Different Water Concentrations

The activity of many water-soluble monomeric and oligomeric enzymes, as well as that of membrane enzymes entrapped in reverse micelles has been studied. Thus, numerous studies have been made to establish the kinetics and characteristics of enzymes in such conditions; the effect of water concentration on enzyme activity has received particular attention. In fact, almost the totality of the reports on the activity of enzymes entrapped in reverse micelles includes a figure that describes activity as function of Wo or water concentration. At first sight, the general picture that emerges from the data with many different enzymes may appear bewildering. Often the curve is bell shaped with a maximum, or levels off after a particular water concentration is reached; curves in which activity increases with water concentration until the system becomes unstable have also been reported. Furthermore, oscillations in the plot of activity vs. water concentrations have been observed repeatedly. It is also noteworthy that some enzymes show catalytic rates that are significantly higher than in standard aqueous systems, that is, the so-called superactivity.

B. The Bell-Shape Profile

The bell-shape type of curve with a maximum has been mostly studied with enzymes entrapped in reverse micelles formed with AOT, but the profile has also been observed in micelles formed with CTAB (Katiyar et al., 1988, 1989; Kumar et al., 1989; Mulimani and Lalitha, 1993); thus, it is not exclusive of a given micellar system. The precise reason of why some enzymes exhibit maximal activity at a particular water concentration is not clear, and some groups have raised a word of warning regarding some of the data (Walde et al., 1992; Bru and Walde, 1991; Bru and Walde, 1993). In a relevant study, Mao and Walde (1991) explored if the substrate could determine the profile of enzyme activity at various Wo; they examined the activity of α-chymotrypsin with six different substrates and found different activity profiles with

different substrates, thus indicating that at least with α-chymotrypsin the same enzyme could exhibit different responses as function of water concentration. Likewise, it has been reported that the profile of activity vs. water concentration results from pH changes in the micellar water pool that occur after product formation (Walde et al., 1992).

Furthermore, Bru and Walde (1991) described that depending on kinetic interpretation of the data, it is possible to obtain different profiles. These authors determined the Km and kcat of α-chymotrypsin at various Wo; they found that both, kcat and Km increase with Wo; they made the point that the plot of kcat vs. Wo was not bell shape, whereas the plot of initial velocity, at supposedly saturating substrate concentrations, resulted in a bell shape profile. Along this line, Marzola et al. (1991) found that the rate of the deacylation step of α-chymotrypsin at various Wo did not exhibit a bell shape profile. As inferred from the overall data and the analysis by Bru et al. (1995), it is evident that careful analysis of solid kinetic data is fundamental for ascertaining if an enzyme entrapped in reverse micelles indeed exhibits a bell-shape profile.

In the context that some enzymes entrapped in reverse micelles exhibit a bell-shape curve, there is a provocative proposal that has been substantiated experimentally in various reports (*vide infra*). The proposal is that the dimensions of the water pool at which catalysis is maximal coincides with the dimensions of the enzyme. In their initial works, the researchers (Martinek, 1989; Khmelnitsky et al., 1989) determined the activity of homotetrameric lactate dehydrogenase, entrapped in reverse micelles formed with AOT as function of Wo. They observed that the enzyme exhibits a profile with four peaks. The dimensions of the micelles at the four maxima coincided with the dimensions of the enzyme at various states of monomer association. It is noted, however, that in other studies the same enzyme entrapped in reverse micelles formed with CTAB (Fernandez-Velasco et al., 1992) or AOT (Khmeltnitsky et al., 1993a) did not show a peak of maximal activity when examined at various water concentrations. However, the results of time-resolved polarized fluorescence studies of lactate dehydrogenase entrapped in reverse micelles showed that lactate dehydrogenase could undergo dissociation in reverse micelles (Khmeltnitsky et al., 1993b).

Activity peaks were also observed with γ-glutamyl transferase (Kabanov et al., 1989, 1990) and alkaline phosphatase from intestinal mucosa (Kabanov et al., 1991). With both enzymes, the activity peaks appeared in regions in which the size of the water pool of the micelles was equivalent to that of the dissociated monomers; the authors confirmed their data by ultracentrifugation studies. Likewise, Tang and Chang (1996) examined the activity of octopus glutathione transferase. This homodimeric enzyme exhibited a maximum in plots of activity vs. Wo; the size of the water pool at that point corresponded to the estimated dimensions of the monomer. Cross-linking of glutathione transferase entrapped in reverse micelles was performed, and the products were analyzed by SDS/PAGE. It is significant that no dimers were observed, suggesting that after entrapment, dissociation took place.

Similar studies were carried out by Chang et al. (1994) with tetrameric malic enzyme from pigeon liver. The activity profile of this enzyme as function of Wo showed two peaks that appeared at the points in which the water pools of the micelle correspond to those of the monomer and dimer; the authors again used cross-linking in reverse micelles to estimate the level of monomer association.

In what seems to be a critical test of the hypothesis, Kabanov et al. (1991) determined as function of Wo the activity of

α-chymotrypsin, and that of α-chymotrypsin that had been cross-linked to a hexameric structure. With both enzymes, a peak of maximal activity was observed, but it is significant that in the cross-linked enzyme, the peak shifted to a higher Wo, which according to the considerations of the authors corresponded to that of micelles with a water pool that had the dimensions of the hexamer.

The hypothesis that the highest activity of enzymes entrapped in reverse micelles occurs when their dimensions coincide with those of the water pool certainly merits attention. However, it cannot be generalized to all enzymes; moreover, with closely related enzymes, different results have been obtained. For example, with alkaline phosphatase from intestinal mucosa (Kabanov et al., 1991), a peak of maximal activity appeared in regions in which the size of the micelle corresponded to the size of dissociated monomers. However, Gonnelli and Strambini (1988) observed that alkaline phosphatase from *Escherichia coli* entrapped in reverse micelles also exhibited a bell-shape profile, but the peak was at a Wo in which the size of the micelle was four times larger than that of the enzyme. In contrast, in the studies of Chang and Shiao (1994) with alkaline phosphatase from human placenta, the curves of kcat vs. Wo did not reveal an activity peak, that is, kcat increased linearly with Wo.

C. Superactivity

In addition to the bell-shape profile, there is another phenomenon that has often been observed with enzymes entrapped in reverse micelles. Some enzymes exhibit activities that surpass those of the enzymes in solution. Superactivity has been observed with α-chymotrypsin (Menger and Yamada, 1979; Barbaric and Luisi, 1981), dihydrofolate reductase (Katiyar et al., 1989), peroxidase (Martinek et al., 1982), wheat germ acid phosphatase (Klyachko et al., 1986), and potato acid phosphatase (Lalitha and Mulimani, 1996). However, Luchter-Wasylewska (1996) did not observe superactivity with human prostatic acid phosphatase.

In regard to superactivity, Gonnelli and Strambini made an interesting suggestion. As noted, these authors (1988) found that the kcat of alkaline phosphatase exhibited a bell-shape profile with Wo, but they also observed that at the optimal water concentration, kcat was about five times higher than in water. The authors suggested that in reverse micelles catalytic activity could depend on the activity of micellar water.

A priori, it would seem that the ascertaining of the differences, and in particular the unusual behavior that enzymes exhibit in reverse micelles, will yield further important information on the factors that affect enzyme catalysis. The suggestion that the activity of water bears strongly on enzyme activity seems particularly appealing, especially if one takes into account the growing information on how water activity affect the kinetics and thermodynamics of biological reactions (see below).

D. Membrane Enzymes in Reverse Micelles

It has been considered by various authors (Martineck et al., 1986; Gallay et al., 1987; Nicot et al., 1985; Vacher et al., 1989) that reverse micelles mimic biological membranes. Thus, it is rather peculiar that only a few membrane enzymes have been studied in reverse micelles. It is not that these enzymes cannot be entrapped in reverse micelles; indeed, cytochrome c oxidase from mitochondrial membranes, one of the most

complex multisubunit enzymes known (Tsukihara et al., 1995), has been incorporated into reverse micelles in a state amenable for spectroscopic and enzymatic studies (Escamilla et al., 1989; Bona et al., 1990). Rhodopsin (Darszon et al., 1978), the reaction center of *Rhodospirillum sphaeroides* (Schonfeld et al., 1980), the Ca^{2+}-ATPase of sarcoplasmic reticulum (Ferreira and Verjovski-Almeida, 1989; Barrabin et al., 1993), and mitochondrial and chloroplast ATPases (Garza-Ramos et al., 1990; Kernen et al., 1997) have also been incorporated into reverse micelles in an active state.

The studies with cytochrome c oxidase were made in systems formed with asolectin. The spectrophotometric recordings by Escamilla et al. (1989) and Bona et al. (1990) showed that in these conditions, the enzyme could be reduced by ascorbate or tetramethyl-*p*-phenylenediamine, and that it could catalyze oxygen uptake, albeit at low rates. Cytochrome oxidase entrapped in reverse micelles reacted with cyanide, yielding spectroscopic complexes that were similar to those observed in water. The enzyme also catalyzed the transfer of electrons to oxygen, although at low water concentrations the rate of oxygen uptake was orders of magnitude lower than in water. In regard to the latter observation, it is relevant that reduced cytochrome oxidase did not react with CO, indicating that at low water concentrations there were severe hindrances in electron transfer from heme a to heme a_3.

The Ca^{2+}-ATPase of sarcoplasmic reticulum has also been incorporated into reverse micelles formed with AOT (Ferreira and Verjovski-Almeida, 1989) and asolectin (Barrabin et al., 1993). In the former studies, it was found that steady-state and time-resolved fluorescence of intrinsic tryptophans was affected by the water content of the system, but apparently no catalytic activity was observed. In contrast, the enzyme entrapped in a phospholipid system exhibited an activity that increased with water concentration. Barrabin et al. (1993) also determined the formation of acyl-phosphate (an intermediate of the catalytic cycle) with ATP and inorganic phosphate as substrates; it is mechanistically important that the ATP-dependent formation of acyl-phosphate took place at water concentrations lower than when phosphate was the substrate.

The arrangement of membrane enzymes in reverse micelles is not known; nonetheless, in view of the structure that enzymes have on biological membranes various models have been proposed (Darszon and Shoshani, 1992). The models generally assume that the hydrophobic part of the proteins lies in the apolar solvent, and that the portion of the protein that contacts the water phase in biological membrane is in the water pool of the micelle. In these models, the micellar wall would mimic the phospholipid surface of the membrane.

V. CHARACTERISTICS OF CATALYSIS BY ENZYMES DISPERSED IN ORGANIC SOLVENTS

In 1966 and 1967 Dastoli, Musto, and Price reported that chymotrypsin and xantine oxidase were active in organic solvents. These important observations were not pursued until nearly 20 years later. Zaks and Klibanov (1984, 1985) gave life to the field when they reported that lipase dispersed in organic solvents exhibited important catalytic activity in systems in which the predominant component was an organic solvent; moreover, the authors made the dramatic finding that lipases could carry out catalysis at temperatures of 100°C. Further work showed that in such conditions many different enzymes exhibit catalysis. For ex-

ample, subtilisin, α-chymotrypsin (Zaks and Klibanov, 1988b, Westcott and Klibanov, 1993), lipases (Zaks and Klibanov, 1985), yeast alcohol dehydrogenase, polyphenol oxidase, horse liver alcohol dehydrogenase (Zaks and Klibanov, 1988a) and horseradish peroxidase (Ryu and Dordick, 1992), and alkaline proteases (Tawaki and Klibanov, 1992) are active in organic solvents.

It is important to point out that the enzymes that were studied differ markedly in structure and catalytic mechanisms. Thus, suggesting that many, and perhaps all, enzymes have the capacity to carry out catalysis under conditions that at first sight would appear catastrophic for enzyme action and structure.

A. Kinetics

In the range of substrate concentration that could be assayed, the enzymes dispersed on organic solvents exhibited Michaelis-Menten behavior. However, as illustrated by the work of Ryu and Dordick (1989, 1992), several factors were found to exert strong influence on the expression of catalysis. These investigators examined in various solvents the activity of horseradish peroxidase deposited in nonporous beads with various substrates. Their results showed that catalytic efficiency increased as substrate hydrophobicity increased. Furthermore, they also observed that this effect was enhanced as the hydrophobicity of the solvent increased.

Regarding the kinetics of enzymes in organic solvents, Wangikar et al. (1993) determined the effect of solvents on the kinetics of subtilisin BPN′ in which an active site Gly was substituted for Asn. Remarkably, they saw that in different solvents the enzyme exhibited differences of catalytic rates of more than two orders of magnitude. They also made the important observation that in dry tetrahydrofuran, acylation is rate-limiting, whereas in the same solvent with 2% water, deacylation became rate limiting. This opened the question of whether water affects the kinetics of the enzyme at the catalytic site. Significantly, Chang and Shiao (1994) showed that the entrapment of alkaline phosphatase in reverse micelles causes a shift of the rate-limiting step.

In subsequent work, Wangikar et al. (1995) pointed out that although the energetics of hydrophobic interactions between substrate and solvent can be determined through solvation calculations, the contribution of hydrophobic interactions between the substrate and the enzyme are difficult to define due to the uncertainties in the hydrophobic state of the active site. To this end, the authors determined the free energy of activation for various *N*-acetyl-L-amino acid ethyl ester substrates that differ in amino acid side chain hydrophobicity in three enzymes, that is, wild-type subtilisin BPN′ and two mutants in which a glycine (166) at the active site (the S1 binding cleft) was substituted by either an alanine or a valine. According to the authors, the substitutions make the catalytic site more hydrophobic and exert only small steric influences on kcat/Km. Thermodynamic treatment of the data showed that in aqueous media, the mutations affected enzyme specificity, whereas in hydrophobic solvents the mutations strongly affected the transition state. Hence, their data indicated that in the expression of catalysis by enzymes dispersed in organic solvents, there is a fine balance between at least three components: the polarity of the substrate, the solvent, and the catalytic site. The authors stressed that by adjustments in these parameters it is possible to make quantitative predictions on how to control catalytic rates and substrate specificity.

B. Specificity

One of the most remarkable findings with enzymes dispersed in organic solvents is that depending on the solvent, some enzymes manifest changes in substrate specificity. In an extensive list of related works, Klibanov and colleagues (reviewed in Westcott and Klibanov, 1994) have documented that selectivity of some enzyme for a given substrate may be drastically altered by the solvent in which the enzyme is placed. For example, Tawaki and Klibanov (1992) determined the rates of transesterification reaction of the D and L forms of N-Ac-Phe-Oet with propanol catalyzed by an alkaline serine protease from *Aspergillus oryzae* in different solvents. Their results showed that the ratio of rates with the L and D forms varied with the solvent in a range that went from 7.1 with acetonitrile to 0.19 with tetrachloromethane. Similar results have been reported for the subtilisin catalyzed transesterification reaction of two different susbtrates with propanol (Westcott and Klibanov, 1993), and, more recently, for the γ-chymotrypsin-catalyzed transesterification reaction of the D and L forms of 3–hydroxy-phenylpropionate with propanol (Westcott et al., 1996). These findings together with those of Gololobov et al. (1992) and Calvet et al. (1993) who reported that the specificity of alpha chymotrypsin is drastically different in water than in a mixture of acetonitrile, dimethyl formamide, and water (4.4% v/v), convincingly indicate that solvents do modify enzyme specificity.

In this context, studies with pyruvate kinase in reverse micelles are noteworthy. In aqueous media, K+ is an essential activator of pyruvate kinase (Kachmar and Boyer, 1953); Na+ is very weak activator. Ramirez-Silva et al. (1997) observed that in pyruvate kinase entrapped in reverse micelles formed with CTAB, Na+ induced a strong activating effect, and that the selectivity of the enzyme for K+ and Na+ was smaller that in solution. Following a previous proposal of Miller (1993), the authors suggested that in water, the high discrimination between K+ and Na+ is consequence of the higher energy required for desolvation of Na+, and hence its partition into its site in pyruvate kinase; in the limited water environment of reverse micelles, the energy barrier would be lower, and thus Na+ is an effective activator.

A present day challenge is to make quantitative predictions on how to adjust the behavior of the enzyme toward a given substrate by solvent design. Work along this line has been reported (Westcott and Klibanov, 1993; Westcott and Kilbanov, 1994; Wangikar et al., 1995; Ke et al., 1996; Westcott et al., 1996; Klibanov, 1997). From the analysis of the experimental data, and assuming that the catalytic site is not modified by solvent, it would appear that solvation energy contributes strongly to the partition of different substrates into the catalytic pockets. Along this line, it is relevant that spontaneous synthesis of pyrophosphate and ATP, two "high-energy" compounds, can be catalyzed by soluble mitochondrial ATPase when the partition of inorganic phosphate into the catalytic site is facilitated by lowering the activity of water with dimethyl sulfoxide (Tuena de Gómez-Puyou et al., 1993).

Therefore, it would seem that the rational manipulation of solvents to alter both the partition of substrates into the catalytic site and the equilibrium constant of the reaction, in conjunction with modification of catalytic sites by site-directed mutagenesis, could be powerful tools to manipulate enzyme action.

VI. WATER, ENZYME FLEXIBILITY, AND CATALYSIS

As noted, there are important differences in the response to water concentration in

enzymes dispersed in organic solvents and when entrapped in reverse micelles. Nevertheless, there is a water concentration in which independently of the nature of the experimental system, all enzymes exhibit an identical characteristic. When water concentration is below monolayer coverage, or at very low Wo (3 or below) in reverse micelles, the activity of enzymes is hardly detectable, or many times lower than in conventional aqueous media. Paradoxically, it is in these conditions in which research has given fundamental information on basic enzymology and has opened new panoramas in biotechnology.

The low activity or the lack of activity that enzymes exhibit at low water concentrations is not consequence of enzyme denaturation, at least irreversible, as an increase in water brings about the appearance of catalytic activity. Lack of substrate binding would also not seem to account (at least totally) for the phenomenon. For example, the number of competent catalytic sites of crystallized subtilisin suspended in acetonitrile was determined from titrations of the active site serine (Schmitke et al., 1996); it was roughly one half of that observed in solution, albeit the differences in kcat/Km was of the order of 10^7 times. Strambini and Gonnelli (1988) observed that alcohol dehydrogenase entrapped in reverse micelles with a Wo of 11 hardly showed catalytic activity, and yet binding of the coenzyme was similar to that observed with the enzyme in solution. Likewise, triosephosphate isomerase entrapped in reverse micelles with a low water content showed less than 5% of the activity in solution, although binding of a substrate analogue was unimpaired (Garza-Ramos et al., 1994).

A. Enzyme Flexibility

Low catalytic rates at low water concentrations have been ascribed to the low flexibility that enzymes have in such conditions. Indeed, the role of water on the flexibility of enzymes has been instrumental in much of the work carried out with enzymes dispersed in organic solvents (see Klibanov, 1989, 1997; Dordick, 1992). A basic concept in this line of work is that in a low water environment, enzymes are relatively rigid structures that may manifest properties that would not be appreciated when water is in excess. In this respect, the pioneer studies of Rupley et al. (1983) with lysozyme powders at various levels of hydration showed the strong dependence of protein mobility and enzyme activity on water. Moreover, from time domain spectroscopy studies of α-chymotrypsin, Bone (1987) proposed that water-induced flexibility of proteins is consequence of a diminution in the interactions between charged and polar residues of the protein molecule caused by dielectric screening (Bone, 1987). In their work, water was visualized as a plasticizer that facilitates structural flexibility (Bone and Pethig, 1982) by shielding interactions between polar residues.

In solution, enzymes are indeed very flexible structures (Karplus and Macamon, 1983; Reid and Rand, 1997). The importance of the inherent mobility of enzymes becomes clearly evident during catalysis. In a catalytic cycle, enzymes undergo conformational changes when the substrate binds to the active center, when it is transformed into product, and when the latter is ejected into the media. Extensive data now exist on the crystal structure of enzymes, with and without active site ligands, and show that in some enzymes the conformational changes are rather subtle, but in others they are quite large. For example, some enzymes, such as lipase (Brzozowski et al., 1991), triosephosphate isomerase (Wierenga et al., 1991), and lactate dehydrogenase (Holbrook et al., 1972), have lids or loops that close over the active site after substrate binding in move-

ments of several Å. Therefore, in a catalytic cycle, there must be a strong interplay between the solvent and many protein groups (Reid and Rand, 1997).

In full consonance with this idea, Burke et al. (1993) showed that the flipping rate constant of a tyrosine residue of α-lytic protease suspended in organic solvents is 10^3 s^{-1}; whereas in solution it is four orders of magnitude higher. Moreover, EPR measurements of spin-labeled residues of α-chymotrypsin in solvents of different dielectric constant showed that motion increases as the dielectric constant of the solvent increases (Affleck et al., 1992a); the latter authors also carried out molecular simulations and observed that the more affected residues were those in the outer regions of the protein.

Affleck and co-workers (1992b) carried out a parallel study of a transesterification reaction catalyzed by subtilisin Carlsberg and the dynamics of nitroxide spin label bound to the active site serine in tetrahydrofuran. They observed that the progressive addition of water produced an increase in activity that was followed by diminution and nearly complete abolition of catalysis. Significantly, the authors also observed that the increase in catalytic rates coincided with an increase in active site polarity and an enhancement of mobility of the spin label.

Suzawa and co-workers (1995) compared the activity in various organic solvents of immobilized α-chymotrypsin (in glass) with that of the suspended enzyme. In parallel they measured the mobility of a spin label at an active site methionine by electron spin resonance. They found that the immobilized enzyme exhibited a higher activity than the suspended enzyme and a larger mobility of the probe. (It is noteworthy that the authors found a way to improve the catalytic efficiency of enzymes in nonaqueous media, which will be in consonance with the work of Adlercreutz [1991] on the influence of solid supports on enzyme activity.) In addition, Suzawa et al. (1995) observed that the addition of water brought about an increase an activity and either, an increased motion of the probe, or an increase in polarity.

With this in mind, Marzola and co-workers (1991) examined the dynamics of the spin-labeled active site of α-chymotrypsin entrapped in reverse micelles. Their data showed that the label exhibited a lower mobility than when the enzyme was in water. Dorovska-Taran et al. (1993a) examined the intrinsic fluorescence of tryptophan of α-chymotrypsin entrapped in reverse micelles of the AOT type in the very low Wo range. Their data show that relative to the enzyme in aqueous media, at Wo of 0.65, the enzyme exhibited a marked blue shift of its intrinsic fluorescence; according to the authors at this Wo, the enzyme was in an apolar environment and in a frozen state. In this state, the enzyme was largely inactive. As water concentration was increased, activity appeared and the fluorescence spectra shifted to the red. Dorovska-Taran et al. (1993a) also pointed out that the amount of water at which catalytic activity was induced in reverse micelles and in the enzyme suspended in tetrahydrofuran (Affleck et al., 1992b) was in the same range.

Hence, the relationship that exists between protein mobility, activity, and water has been observed in liophylized protein films, enzymes dispersed in organic solvents, and enzymes entrapped in reverse micelles.

B. Enhancement of Catalytic Activities by Denaturants

On the basis that the activity of enzymes in a low water environment is hampered by constraints in conformational mo-

bility, it was asked if the activity of enzymes in a low water environment could be increased by agents that promote solvent-protein interactions (Garza-Ramos et al., 1992; Fernandez-Velasco et al., 1992). Although a precise definition of the mechanism of action of denaturants is lacking (Tanford, 1980; Pace et al., 1990), it is generally accepted that they favor the exposure of protein groups into the solvent. When the effect of guanidine chloride was tested on six enzymes (heart and muscle lactate dehydrogenase, α-glycerol-phosphate dehydrogenase, hexokinase, inorganic pyrophosphatase, and glyceraldehyde-3–phosphate dehydrogenase), it was found that the denaturant induced a severalfold enhancement of catalytic rates in the former five enzymes (Garza-Ramos et al., 1992). It is pertinent to note that in the enzymes that were studied, the increase of catalytic rates induced by the denaturant guanidine chloride was of different extent, indicating that the activation is affected by the nature of the enzyme. This is more clear with heart and muscle lactate dehydrogenases (Fernandez-Velasco et al., 1992), although the two enzymes are quite similar, the latter is much less sensitive to the denaturant.

Activation of enzymes dispersed in organic solvents could also be achieved by denaturing cosolvents. Alamarsson and Klibanov (1996) observed that dimethyl sulfoxide and formamide produced a dramatic increased in activity of subtilisn, α-chymotrypsin, and lipase; it is relevant that activation by denaturing cosolvents in the three enzymes was of a different extent. The authors concluded that the denaturing cosolvents brought about an increase in the flexibility of the enzyme. It is significant that in reverse micelles, methanol produced strong activation of chloroplast ATPase (Kernen et al., 1997). Thus, it would seem that cosolvents could be used advantageously to increase catalytic rates in low water environments.

C. Factors That Contribute to the Low Activity of Enzymes in a Low Water Environment

All the aforementioned data are in concordance with the idea that in low water milieu the conformational freedom of enzymes is restricted. However, they do not prove that enzyme rigidity is the sole cause of the low activity that enzymes express in low water environments. Schimtke et al. (1996, and see discussion by Klibanov, 1997) addressed the question by comparing the activity of subtilisin in solution with that of cross-linked crystallized subtilisin in acetonitrile in the hydrolysis of *N*-acetyl-λ-phenylalanine ethyl ester. (It is noted that the crystal structure of the cross-linked enzyme is strikingly similar to that of the non cross-linked enzyme [Fitzpatrick et al., 1993, 1994; Schmitke et al., 1997].) The difference in kcat/Km at pH 7.8 of the enzymes in the two conditions was of the order of 10^7 times. From systematic variations of various parameters, they found that three factors contribute to the differences in kcat/Km: a shift of the activity to a more alkaline pH in the enzyme in the organic solvent, an unfavorable effect on the desolvation of the substrate (and in consequence on its partition to the catalytic site), and, finally, a diminished conformational freedom of the enzyme. From experiments on the effect of two solvents with different capacity to form H-bonds, the authors also suggested that the latter barrier for enzyme catalysis was related to the thermodynamic activity of water. If these mechanisms also operate in other enzymes or whether additional factors (Dong et al., 1996) account for the low activity

that enzymes express in low water environments remains to be established.

It is important to acknowledge that extensive efforts are being made to increase the rates of enzyme catalysis in organic solvents (Adlercreutz, 1991; Klibanov, 1997). In this context, it is relevant that Paradkar and Dordick (1994) and Khmelnitsky et al. (1994) reported large increases in activity of enzymes dispersed in organic solvents by salts and surfactants.

From the above data it appears clear that water is instrumental in protein mobility, as well as on other factors that participate in the expression of catalysis. Because enzymes were designed by evolution to work in an aqueous milieu, this conclusion may seem trivial and obvious. However, as shown in the following sections, the analysis and proof of this concept has led to the visualization of many novel and unsuspected characteristics of enzymes.

VII. WATER AND THE RELATIONSHIP BETWEEN STABILITY AND CATALYSIS

Solvation is necessary for protein function; however, excessive solvation may lead to loss of the native protein structure. This is more notable at relatively high temperatures in which many of the weak bonds that maintain the native structure are destabilized and solvated. Therefore, it was rationalized that in a low water environment, enzymes should exhibit a high resistance to thermal denaturation.

A. Thermostability

The latter proved correct. The following enzymes dispersed in organic solvents with low water concentrations were found to exhibit thermostability many times higher than in solution: lipases (Zaks and Klibanov, 1984, 1985), terpene cyclases (Wheeler and Croteau, 1986), chymotrypsin (Reslow et al., 1987), α-amylase (Ashter and Meunier, 1990), ribonuclease, lysozyme (Volkin et al., 1991), and chymotrypsin. Mozhaev et al. (1991) also observed a dramatic thermostability of α-chymotrypsin in nonpolar organic solvents. (This work is of particular interest because the authors claimed that they had been able to solubilize the enzyme in nonpolar solvents.) It is noteworthy that thermostability decreased as the water content of the systems was increased.

Two membrane proteins, cytochrome c oxidase (Ayala et al., 1986) and mitochondrial ATPase (Garza-Ramos et al., 1989, 1990), and the water-soluble triosephosphate isomerase (Garza-Ramos et al., 1994) also exhibited a remarkable thermostability when entrapped in reverse micelles formed with phospholipids and low amounts of water. For example, with 1.3% water, the half-life of the ATPase at 70°C was about 11 h; with 0.03% water the half-life became 96 h (Ayala et al., 1986); and in 100% water, it was less than 1 min.

However, it is noted that a number of reports show the stability of enzymes entrapped in reverse micelles formed with synthetic detergents is lower than in solution (Luisi et al., 1990; Khmelnitsky et al., 1993a; Dorovska-Taran et al., 1993a; Chang and Shiao, 1994), albeit Escamilla et al. (1992) reported that catalase exhibits a high thermostability when entrapped in reverse micelles formed with AOT. Taken together the data in reverse micelles suggest that the nature of the amphiphile is paramount. The nature of the enzyme will seem to play a lesser role, because membrane and water-soluble enzymes exhibit high thermostability in systems formed with phospholipids.

A salient feature in all the enzymes in which thermostability has been documented is that thermostability decreases as the water content in the system is increased. Indeed, the inverse relationship between enzyme thermostability and water concentrations became apparent from the first reports with enzymes dispersed in organic solvents (Zaks and Klibanov, 1984), or entrapped in phospholipid systems (Ayala et al., 1986).

B. Cleavage of Covalent Bonds in Low Water Systems

Thermal denaturation is often accompanied by cleavage of covalent bonds. In fact, in the studies of Volkin et al. (1991) thermal denaturation of ribonuclease in nonane was irreversible as a consequence of deamidation that occurred through a transamidation reaction. Along this line, it is relevant to note that in solution triosephosphate isomerase undergoes thermal denaturation at temperature of 57°C through a process that is accompanied by deamidation. In a low water system formed with phospholipids, the enzyme was more stable and no deamidation was observed (Garza-Ramos et al., 1994). Therefore, in a low water environment enzymes become more resistant to thermal-induced unfolding and, very likely, to cleavage of covalent bonds.

C. The Cost of Catalysis

The enzymes in which thermostability has been documented are markedly different in structure and function, and yet in a low water environment all exhibit a thermostability that is much higher than in standard aqueous systems; it is important to point out that under these conditions the enzymes express low catalytic rates. When the water content of the system is raised, enzymes work faster, but they also become progressively susceptible to thermal denaturation.

The favorable effect of water on catalysis and its detrimental effect on enzyme stability may be rationalized in terms of protein rigidity or flexibility. As the amount of water in contact with the enzyme is progressively increased, there is an enhancement of protein flexibility, and, in consequence, a higher capacity to carry out catalysis. However, the cost of high catalytic rates is paid by a higher sensitivity of the enzyme to thermal denaturation. In the inverse relationship that exists between catalysis and thermostability, the studies of Shoichet et al. (1995) are of particular relevance. These researchers determined in aqueous media the activity and the thermal stability of wild-type lysozyme and mutants in which active site residues were substituted. They observed that in the mutants a diminution or an abolition of catalysis was accompanied by an increase in stability.

However, one does not have to go to nonconventional systems or to site-directed mutagenesis to observe the inverse relationship between catalysis and stability. A comparison of the properties of enzymes from organisms that live in different environments provides a good example of how evolution has considered protein flexibility as a tool for survival. For instance, at room temperature enzymes from thermophiles, relative to their mesophilic counterparts, are rather rigid structures, as a consequence of many apparently subtle, intramolecular interactions (Jaenicke, 1991). At these temperatures, the specific activity of thermophilic enzymes is lower than that of mesophilic enzymes, reflecting their low flexibility at relatively low temperatures. Nonetheless, the inverse relationship between water and enzyme ther-

mostability and catalysis is well illustrated by work in nonconventional systems.

D. Differential Scanning Calorimetry of Enzymes in Organic Solvents and When entrapped in Reverse Micelles

Although in general there are important differences in the stability of enzymes in reverse micelles and when suspended in organic solvents, it is worthwhile to compare some of the data that have been obtained in the two systems, particularly those in which the same enzyme has been studied by the same technique, that is, differential scanning calorimetry.

Ribonuclease dispersed in nonane (Volkin et al., 1991) with 6% water showed an irreversible thermal transition at the remarkably high temperature of 124°C; this progressively decreased with increasing water concentration, reaching value of 61°C in aqueous media. When the stability of the same enzyme entrapped in reverse micelle formed with AOT (Luisi et al., 1990) was examined, it was found that ribonuclease was less stable than in water. However, the researchers observed that once entrapped in reverse micelle, a decrease in water concentration (from a Wo of 25.5 to 11.1) brought about an increase in stability. Similar results were obtained with cytochrome c; the process was also irreversible.

Shastry and Eftink (1996) studied the thermal transition of ribonuclease T1 entrapped in reverse micelles formed also with AOT and various water concentrations. In contrast to the previous studies, these investigators observed that thermal unfolding was reversible at Wo below 12; at the latter Wo, unfolding became irreversible. The latter results would be in concordance with the data of Luisi et al. (1990), who employed micelles with Wo above 11. Shastry and Eftink (1996) indicated that scanning rates, and the time that the sample remained at the highest temperature assayed, were central for reversibility. From the data, Shastry and Eftink (1996) calculated (Tm $\Delta G°$, and $\Delta H°$). At a Wo of 4.6, the values were similar to those in aqueous media; the values decreased with water concentration.

It would have been important if stability in reverse micelles had been determined at lower Wo, particularly at those on which enzymes acquire rigidity, that is, 0.65 (Dorovska-Taran et al., 1993b). However, as pointed out by Luisi et al. (1990), there are technical problems in carrying out differential scanning calorimetry studies of proteins entrapped in reverse micelles at very low water concentrations.

The stability of α-chymotrypsin in isooctane equilibrated at various water activities was also studied by differential scanning calorimetry by Oste-Triantafyllow et al. (1996). The transition temperature shifted to a value 30°C lower when the dry enzyme was hydrated. Heat-induced denaturation was irreversible. Thus, the data of Oste-Triantafyllow et al. agree with that of Volkin et al. (1991).

E. Pressure-Induced Unfolding

Before closing this section, it is pertinent to note that protein denaturation can also be induced by high hydrostatic pressures, and that this may be a powerful tool to determine the thermodynamics of protein folding (Silva and Weber, 1993). To date, no data exist on pressure-induced denaturation in low water media; thus, the work of Oliveira et al. (1994) with the Arc repressor is of particular interest. Pressure-induced denaturation of the Arc repressor was determined at various concentrations of glycerol.

The highest concentration that was studied was 50%; however, extrapolation of their data to zero water led to the conclusion that the repressor would not denature in the absence of water. Thus, this approach may be of potential use for ascertaining the thermodynamics of protein stability in low water systems.

VIII. TRAPPING OF ENZYME CONFORMERS IN LOW WATER SYSTEMS

On the basis that water is an essential component of conformational mobility, it is theoretically possible to stabilize different protein conformations by adjustment of the amount of water in contact with the enzyme. The data that have been obtained with enzymes dispersed in organic solvents or entrapped in reverse micelles show that two general types of conformations may be stabilized in low water systems. One corresponds to enzymes that exhibit particular catalytic properties, and the other to intermediates of biological reactions that in water have relatively short life times.

A. Stabilization of Conformers with Particular Catalytic Properties

Russell and Klibanov (1988) made the following observation. Subtilisin was incubated in aqueous media with a reversible active site inhibitor; subsequently, the mixture was lyophilized, and the powder washed with an organic solvent that effectively removed the inhibitor. Thereafter, the capacity of the enzyme to catalyze a transesterification reaction in octane was measured. The authors observed that in comparison to the control, subtilisin that had been exposed to the inhibitor exhibited a substantially higher activity if the water content of system was kept below 0.012%. The authors interpreted their results as locking of an active conformation of subtilisin that had been induced by the competitive inhibitors and called the phenomenon "enzyme memory".

Following the same rationale, Stahl et al. (1990, 1991) incubated α-chymotrypsin with the anomalous substrate *N*-acetyl-D-tryptophan in aqueous media and precipitated it with propanol. The enzyme was dried and its capacity to form acetyl-D-tryptophan ethyl ester in cyclohexane with increasing water concentrations was determined. The enzyme catalyzed synthesis of the D-ester in a bell shape type of curve with a maximum at about 1.5 m*M* water; with 4 m*M* water hardly any synthesis of the D-ester took place. In the terminology of the authors, the active site was "bio-imprinted" to catalyze synthesis of the D-ester, a bio-imprinting that persisted as long as water was kept at low levels. In contrast, the enzyme imprinted with *N*-acetyl-L-tryptophan did not exhibit the anomalous activity; however, it effectively catalyzed synthesis of *N*-acetyl-L-tryptophan ethyl esther at rates that increased with water concentration. These exciting results exemplify that the increased flexibility induced by increasing water concentrations leads to an increase of "normal" catalysis, but that it also provokes loss of the imprinting that allowed the enzyme to catalyze the "normal" reaction.

B. Stabilization of Intermediates of Biological Reactions

It is relevant that in the aforementioned works, the enzymes were frozen in particular conformations; however, the enzymes

still had sufficient flexibility to support catalysis. Thus, it may be asked why, if there was flexibility to support catalysis, the enzymes did not revert to the "normal" catalytic conformation. This may indicate that to undergo different conformational changes, enzymes require different water concentrations. This question was addressed by Barrabin et al. (1993) with the Ca^{2+}-ATPase of the sarcoplasmic reticulum. The enzyme catalyzes the hydrolysis of ATP in a process that is coupled to the transport of Ca^{2+} across the membrane. An acyl-phosphate at the catalytic center is an intermediate of the hydrolytic reaction; this can be formed from either phosphate or ATP (de Meis et al., 1980). The Ca^{2+}-ATPase entrapped in reverse micelles formed with phospholipids catalyzed the hydrolysis of ATP through a process that increased with water concentration. The authors measured the amount of the acyl-phosphate that was formed when ATP or phosphate was included in the system. They found that with ATP the amount of water required for acyl-phosphate formation was lower than with phosphate, indicating that different steps of the catalytic cycle require different water concentrations.

In another membrane enzyme, Escamilla et al. (1989) showed that in reverse micelles of the phospholipid type with low water content, the catalytic cycle of cytochrome c oxidase could be arrested at the level of electron transport between hemes a and a_3. In addition to the trapping of conformers of catalysis, it is also possible that intermediates of the protein folding pathway could be estabilized in reverse micelles. In fact, Lenz et al. (1995) reported that insulin acquired a molten globule conformation after its entrapment in reverse micelles.

Another line of evidence that indicates that intermediates with short-life times can be stabilized in reverse micelles by adjustments of water concentrations derives from experiments that showed that the catalytically active dimer of triosephosphate isomerase was formed after entrapment of unfolded monomers in reverse micelles formed with CTAB (Garza-Ramos et al., 1992; Fernandez-Velasco et al., 1995). The rate at which catalysis appeared in reverse micelles increased with protein concentration, and it was thus considered that the appearance of catalysis depended on the formation of the dimeric structure. Similar data in standard aqueous systems had been reported (Waley, 1973; Zabori et al., 1980); however, in reverse micelles at low water concentrations the monomers could be trapped for significantly long times in a state competent for formation of active dimers; in this state a rise in water concentration led to formation of the active dimer. However, data of fluorescence energy transfer between fluorescence-labeled monomers (Sepulveda-Becerra et al., 1996) did not correlate with appearance of activity. Fernandez-Velasco et al. (1995) indicated that at low water concentrations there was a kinetic barrier in the formation of dimers from the individual monomers.

All the aforementioned observations indicate that different protein conformations may be trapped by adjustments in water concentration. This may have profound implications on biotechnology and basic protein science, in the sense that an enzyme conformer competent for a given reaction may be stabilized and used for bioconversions, and also because intermediates with a short life-time in aqueous media may be stabilized and characterized.

IX. INTERACTIONS AND DISSOCIATION OF PROTEINS IN REVERSE MICELLES

Protein-protein interactions are fundamental in biology. In multisubunit enzymes

these interactions are central in the expression and regulation of their activity. For example, it has often proposed that in some oligomeric enzymes, the monomers that conform them are catalytically inactive, even though only in a few occasions have the monomers at various levels of association been estabilized (Jaenicke et al., 1981). In this context, various reports with oligomeric enzymes entrapped in reverse micelles look particularly promising. It is also relevant that in reverse micelles, complex reactions such as association of macromolecules have also been explored and some data that have been obtained may provide information into the molecular events that occur in biological phenomena.

A. Association of Macromolecules in Reverse Micelles

From many reports, it is well established that in reverse micelles interaction between macromolecules readily takes place. Hanley et al. (1991) observed that phage lambda can be solubilized in reverse micelles, and that in such conditions it could be cleaved by Hind III. It was also found that the rate and sites of cleavage were affected by the nature of the surfactant. Likewise, Bru and Garcia-Carmona (1991) examined the interaction of trypsin with the soybean trypsin inhibitor in reverse micelles formed with AOT. Interaction was judged by the inhibition of catalytic activity with *N*-α-benzoyl-L-arginine ethyl ester as substrate. The inhibitor effectively inhibited trypsin activity, albeit at rates lower than in standard aqueous mixtures. The low rate of interaction was ascribed to a relatively slow rate of fusion between protein-filled micelles. Their results were later confirmed in micelles formed with CTAB (Fernandez-Velasco et al., 1995). Along the same line, Fadnavis et al. (1993) found that in reverse micelles of the AOT type, alpha-chymotrypsinogen was activated by trypsin in a process that depended on water concentration. However, they found that at the optimal water concentration, the rate of activation was faster than in solution, which appears to be at variance with the data of Bru et al. (1991). Nevertheless, the two reports indicate that protein-protein interactions can take place in reverse micelles.

Likewise, Groom et al. (1990) observed that Fab fragments of two monoclonal antibodies raised against bovine myelin basic protein bound to a synthetic peptide with the sequence of the myelin basic protein with affinities similar to those observed in water. With respect to antibody-antigen interactions in reverse micelles, Matveeva et al. (1996) described a fluoroimmunoassay method in reverse micelles for determination of water-insoluble compounds.

Nicot et al. (1993) also examined the cleavage of the myelin basic protein entrapped in reverse micelles by trypsin, cathepsin D, and *Staphylococus aureus* V8 protease. Cleavage was observed in reverse micelles, albeit differences in the site of cleavage were observed when the reaction was carried out in standard aqueous media and reverse micelles. The authors explained the difference as a consequence of different conformations that myelin basic protein acquires in reverse micelles and in 100% water. The overall work of the group in Paris on myelin basic protein (reviewed by Nicot and Waks, 1995) provides a fine example of how reverse micelles as model systems can provide insights into the arrangements that some proteins may acquire within biological structures.

B. Dissociation of Oligomeric Enzymes in Reverse Micelles

In Section IV.B, it was mentioned that some oligomeric enzymes entrapped in re-

verse micelles exhibit peaks of activity at water concentrations in which the dimensions of the micellar water pool coincide with the size of the subunits that conform the oligomer. It was noted that for some enzymes, data from different laboratories do not seem to be in agreement. It should now be added that the claims that the monomers of oligomeric enzymes entrapped in reverse micelles are catalytically active would also appear to be in conflict with data in standard aqueous media that generally show that the monomers are catalytically inactive. For example, monomers (as evidenced from an activity peak at the corresponding Wo and ultracentrifugation data) of alkaline phosphatase are catalytically active in reverse micelles (Kabanov et al., 1991, but see Chang and Shiao, 1994), whereas in water the monomers are inactive (McCracken and Meigen, 1980).

Another enzyme of interest is γ glutamyl transferase. This enzyme is formed by a heavy (Mr = 54,000) and a light subunit of Mr = 21,000 (Gardell and Tate, 1981). Kabanov et al. (1989, 1990) entrapped the enzyme in reverse micelles and from activity data they concluded that the enzyme had dissociated into its constituent subunits; they were able to separate the two monomers by centrifugation and found that individual monomers could carry out catalysis. Because the active site lies between the two subunits, the results in reverse micelles are rather remarkable. If confirmed, the data of Kabanov et al. (1989, 1990, 1991) could have profound implications.

In this context it is relevant that Salom et al. (1992) described a novel method to separate different peptide conformational species in reverse micelles. After entrapping gramicidin A in reverse micelles, the authors were able to quantitate by high-performance liquid chromatography, the population of gramicidin molecules that existed as dimers or monomers. This methodology could have a high potential in micellar enzymology, for example, to probe if there is dissociation of heterooligomers in reverse micelles.

The importance of the aforementioned data in reverse micelles should be evaluated considering that extensive work is being done to understand the structural arrangements that monomers undergo after association to yield an active oligomer (Green et al., 1995; Thanki et al., 1997). Therefore, and regardless of current uncertainties and apparent contradictions between data in solution and in reverse micelles, it seems worthwhile to explore if indeed reverse micelles could be a way to determine the structural differences between active and inactive monomers.

X. STRUCTURE OF ENZYMES IN REVERSE MICELLES

When one observes catalysis by an enzyme placed in nonconventional systems, the natural question that comes to mind is whether in such conditions the enzyme has, or does not have, the structure of the enzyme in solution. In either case, for full comprehension of enzymes it is imperative to determine and compare their structures in the two conditions.

In a recent review Nicot and Waks (1995) made an excellent analysis of the structural features of enzymes entrapped in reverse micelles. They made emphasis on lipases, myelin basic protein, lysozyme, cytochrome c, liver alcohol dehydrogenase, and serine proteases. Thus, only some of the more general and recent data are described. Because solutions of reverse micelles with and without proteins are optically transparent, spectroscopic techniques have been the method of choice for studying protein structure in reverse micelles (Voss et al., 1987;

Verhaert et al., 1992); ligand binding has also been employed to determine structural features of proteins entrapped in reverse micelles (Desfosses et al., 1991; Desfosses et al., 1992).

Circular dichroism (CD) in the far and near UV has often been used to determine the secondary and tertiary structures of proteins entrapped in reverse micelles. A problem in these measurements, particularly with CTAB, is the strong light absorption of surfactant molecules in the region of 190 to 240 nm. Thus, it is of practical importance that Vehaert et al. (1992) showed that micelles formed with cetyltrimethylammonium chloride (CTAC) instead of CTAB can be used to obtain structural information by far UV CD.

Steady-state fluorescence yields information in the environment of the fluorophore as evidenced from shifts of fluorescence maxima or changes in quantum yield. Likewise, because proteins are dynamic entities with fluctuations in times of nanoseconds or seconds, time-resolved fluorescence provides information of whether the reporter group exhibits restricted motion and undergoes conformational substates. Therefore, these two techniques have been employed repeatedly to probe the dynamic structure and the environment of the fluorophore.

In many of these works, the fluorophore has been an intrinsic tryptophan (Marzola and Gratton, 1991; Davis et al., 1996; Shoshani et al., 1994; Visser et al., 1994; Shastry and Eftnik, 1996). However, fluorophores that have been bound covalently to active sites, and other regions of the protein have also been used (Dorovska-Taran et al., 1993b; Lenz et al., 1995). The phosphorescence lifetime of an intrinsic tryptophan in the NAD binding of horse liver alcohol dehydrogenase has been used (Strambini and Gonelly, 1988) to determine the dynamics of that site, and by the same technique Gonnelli and Strambini (1988) determined the dynamics of alkaline phosphatase entrapped in reverse micelles. Agents (such as acrylamide and carbon tetrachloride) that quench the fluorescence of model compounds, or that of intrinsic tryptophan have been employed to probe into the characteristics of the region in which the fluorophore is located (Davis et al., 1996).

With respect to the techniques that have been used to probe the structure of proteins entrapped in reverse micelles, it is noteworthy that Qinglong et al. (1994) were able to obtain Fourier transform infrared spectra of α-chymotrypsin entrapped in reverse micelles of the AOT type (Wo between 6 and 10). Their data showed that entrapment produced a loss of α-helix and β-sheet and an increase of random structure.

On the basis that reverse micelles mimic biological membranes, some studies have been made on the structural arrangements that somatostatin (Bhattacharyya and Basak 1995), two melanocyte-stimulating hormones (1993), and insulin (Lenz et al., 1995) undergo when they are transferred to reverse micelles. The idea behind the latter works is that the structure that the hormones acquire in reverse micelles could represent conformations that may exist during biological phenomena. This idea was originally proposed by Gallay et al. (1987) in their studies with adrenocorticotrophin and glucagon entrapped in reverse micelles of the AOT type. They observed that in reverse micelles, the peptides exhbited restrictions of the internal motion of intrinsic tryptophan. It is also noteworthy that Singh and Aruba (1995) used reverse micelles to probe the environment of the retinal Schiff base in bacteriorhodopsin. It should be noted that in the studies with hormones, reverse micelles were used as biological mimetic systems and yet reverse micelles were formed with commonly used synthetic surfactants; albeit Lenz et al. (1995) also made use of reverse

micelles formed with α-L-1,2–dioctanoyl-phosphatidyl choline.

In another respect, the data of Lenz et al. (1995) are rather provocative; the authors observed that insulin labeled with a fluorescent probe entrapped in reverse micelles acquired the conformation of a molten globule, that is, a main chain similar to that observed in solution, which nonetheless exhibited a weak circular dichroism spectrum in the near UV, reflecting absence of quaternary structure. Their findings also substantiate the idea that intermediates in the formation of native proteins may be stabilized in reverse micelles. Therefore, the overall results suggest that indeed reverse micelles may be a powerful tool to enquire into parameters that in biological systems are difficult to determine.

XI. STRUCTURE OF ENZYMES IN ORGANIC SOLVENTS

There seems to be a generally accepted explanation for the general characteristics of catalysis and thermostability of enzymes in low water environments. However, one can ask if in different solvents the enzyme acquires different structural features that could account for differences in catalysis. It is too early to offer a general answer. However, Burke et al. (1989) examined the hydrogen-bonding of an active site histidine (labeled with 15N) of α-lytic protease by high-resolution solid state NMR in water and organic solvents. Their spectra showed that the geometrical arrangements of the active site residue were not perturbed by the organic solvent. Burke et al. (1992) also examined the active center of α-chymotrypsin by solid NMR spectroscopy after lyophilization and after suspending the powder in organic solvents. They observed that lyophilization disrupted the active centers of a portion of the enzymes; further disruption occurred when the enzyme was suspended in solvents that are known to remove water from the enzyme, but not when suspension was carried out with hydrophobic solvents. On the other hand, Dong et al. (1996) determined the secondary structure of subtilisn and α-chymotrypsin by infrared spectroscopy; in reference to the enzymes in solution, enzymes that had been lyophilized and suspended in organic solvents showed an altered secondary structure.

Therefore, there is a lack of a precise molecular explanation for the particular characteristics that enzymes acquire in organic solvents. This is especially needed when dealing with changes in substrate specificity (Westcott and Klibanov, 1994; Zaks and Klibanov, 1986; Gololobov et al., 1992; Calvet et al., 1993; Tawaki and Klibanov, 1992; Stahl et al., 1990; Stahl et al., 1991), and enzyme memory (Russell and Kilbanov, 1988) or bioimprinting (Stahl et al., 1991). Therefore, the reports that probed the molecular structure of enzymes in organic solvents were most welcome.

A. Molecular Dynamics

The impact of organic solvents on enzyme structure has started to be examined by molecular dynamics simulations. Hartsough and Merz (1993) suggested that the flexibility of proteins in organic solvents does not change, albeit they also indicated that hydrogen bonds and other electrostatic interactions are stronger in the organic solvents. Simulations of lipase from *Rhizomucor miehei* in water, methylhexanoate, and cyclohexane were carried our by Peters et al. (1996). Differences were observed in the three solvents, that is, the protein seemed to "shrink", and there was an increase in the number of hydrogen bonds. Significantly,

they saw that methyl hexoanoate penetrated the active site groove.

B. Crystal Structures

As enzymes are not soluble in apolar solvents (at least in those solvents that maintain their catalytic properties, but see Mozhaev et al., 1991), crystallization in such conditions does not seem feasible. Therefore, enzymes that had been crystallized in aqueous solution were soaked in organic solvents. In the work of Fitzpatrick et al. (1994), we are told that the exposure of crystals of subtilisin Carlsberg to 50% acetonitrile led to cracking. Therefore, the authors carried out cross-linking of subtilisin that had been crystallized in water, followed by soaking in anhydrous acetonitrile (Fitzpatrick et al., 1993, 1994). The crystals were stable and diffracted at a resolution of 2.3 Å. This methodology was also used to ascertain the influence of acetonitrile on the crystal structure of cross-linked porcine pancreatic elastase (Allen et al., 1996). In contrast to subtlisin and elastase, Yennawar et al. (1994, 1995) found that non-crosslinked crystals of γ-chymotrypsin were stable in hexane.

The data of Fitzpatrick et al. (1993, 1994) show that the polypeptide chains of subtilisin in acetonitrile and water are markedly similar; some shifts of active site residues took place, but these were rather minor. It was also observed that only a portion of the water molecules were removed by acetonitrile, and that acetonitrile occupied positions in which no water was visualized in crystals that had not been exposed to acetonitrile. The possibility that alterations of subtilisin induced by organic solvents could be prevented in a cross-linked enzyme was considered unlikely in view of the similarities of the B factors in the two enzymes in water. On the other hand, the B factor of cross-linked subtilisin in hexane was lower, which indicated that in acetonitrile the enzyme acquired rigidity.

In a continuation of the work with subtilisin, Schimtke et al. (1997) examined the crystal structure of the enzyme in the presence of dioxane and compared it with that in acetonitrile. Significantly, they found that the structures in acetonitrile, dioxane, and water were markedly similar. In view of the observations that subtilisin exhibits distinct catalytic activities in different solvents (Ke et al., 1996; Westcott et al., 1996), it is significant that hardly any changes were detected at the active site. The authors also detected dioxane molecules that interact with the protein, and that, in comparison to the data with acetonitrile, different water molecules were replaced by two solvents.

The data of Allen et al. (1996) on crystallized elastase showed that the overall structure of the enzyme was not modified by soaking with acetonitrile. In their work, the main issue was to determine in the crystal structure of elastase, sites in which there was interaction of acetonitrile molecules with the protein. According to the authors and Ringe (1995), these sites may represent potential targets for drug design. In the crystal, nine acetonitrile molecules were observed, two of which were near the active site.

The work of Yennawar et al. (1994, 1995) was specifically aimed to at answering the question of whether there are structural alterations in γ-chymotrypsin induced by hexane, which could account for changes in substrate specificity (Zaks and Klibanov, 1986; Stahl et al., 1990, 1991; Tawaki and Klibanov, 1992). Similarly to the results of Fitzpatrick et al. (1993, 1994) and Allen et al. (1996), the backbone of γ-chymotrypsin in hexane showed no major differences with the enzyme in water. Neverthe-

less, it was observed that some side chains underwent rearrangements, particularly in the regions where hexane molecules appeared. Hexane molecules were found to replace some water molecules, but other hexane molecules appeared in the neighborhood of hydrophobic patches. The investigators also observed that there was strengthening of H-bonds and electrostatic interactions, which could induce rigidity of the enzyme molecule. The catalytic center was carefully examined, no important differences with the enzyme in water were detected.

From the crystallographic data in the three enzymes, it appears clear that organic solvents do not produce large modifications of the three-dimensional structure of the enzyme, nor of its catalytic center. Nevertheless, organic solvents produce changes of the orientation of some residues in the surface of the protein. Also in the three enzymes, there are indications that in organic solvents, the proteins are more rigid than in water which would be in consonance with data on the activity and thermostability of enzymes in organic solvents.

XII. MULTIENZYMATIC COMPLEXES AND CELLS IN NONCONVENTIONAL SYSTEMS

In the last 15 years, research on enzymes in noconventional systems with low water content has gone from the observation of catalysis to the impact that such systems exert on the molecular structure of enzymes. However, the authors feel that this review would not be complete without mentioning, albeit briefly, that active research is also being carried out on the other side of the coin, that is, the level of biological complexity that is functional in nonconventional media. This problem has only been addressed in reverse micelles.

A. Biological Structures in Reverse Micelles

This subject has been reviewed by Pfamatter and co-workers (1992) from historical and conceptual points of view. The results of these studies are remarkable. Successful transfer of the following structures into reverse micelles has been reported: DNA and RNA (Imre and Luisi, 1982), ribosomes (Palazzo and Luisi, 1992), membranes of mitochondria and bacteria, thylakoid membranes (Escobar and Escamilla, 1992; Escamilla et al., 1992; Kernen et al., 1997), whole mitochondria (Hochkoeppler and Luisi, 1989), bacteria (Haring et al., 1985; Darszon et al., 1988), and yeast cells (Hochkoepller and Luisi, 1989; Darszon et al., 1988; Pfammater et al., 1989) Furthermore, Pfafmatter et al. (1990, 1992) reported the transfer of cultured carrot cells into systems formed with water and AOT in hexane and in systems formed with water, asolectin, and decane.

It is important to note that the systems of reverse micelles that contain whole cells are optically transparent and stable, albeit the same amount of cells suspended in water yield a turbid suspension. Pfafmatter et al. (1992) discussed the reasons that may lead to "solubilization" of cells (because the system is transparent, "suspension" of cells may not be the adequate term) in systems that contain an amphiphile, water, and an organic solvent. In the absence of any of the former two, no solubilization took place in the organic solvent.

B. Biological Function

The limit of biological complexity that can be entrapped within a micellar system may or may not have been reached. Nev-

ertheless, the questions that arises is whether integrity of biological structure is preserved in these unnatural environments, and whether some kind of biological function is manifested. Viability may be considered as proof of biological integrity. In this respect, data on the viability of cells that have been transferred into such systems show that some organisms such as *Escherichia coli* seem to be quite sensitive to entrapment, whereas others, such as *Acinobacter calcoaceticus* (Haring et al., 1985) and *Sacharomyces cerevisiae* (Pfammater et al., 1989), remain viable for significantly long times. At this moment, the reasons of why different organisms exhibit different sensitivity to entrapment are not evident.

With respect to whether in such conditions complex systems exhibit some kind of biological function, the experimental results appear promising. Hochkoeppler and Luisi (1989) found that the malate dehydrogenase activity by mitochondria entrapped in reverse micelles was similar to that of mitocondria in water. With yeast cells in a reverse micellar system Fadnavis et al. (1989, 1990) observed peptide synthesis and hydrolysis.

Because respiration in bacteria depends on the coordinated function of several enzymes that lie in the plasma membrane, it is remarkable that membranes of *Bacillus cereus* and *Rhizobium faseoli* entrapped in reverse micelles with asolectin in iso-octane exhibit good rates of cyanide sensitive respiration (Escamilla et al., 1992); oxygen uptake by *B. cereus* entrapped in reverse micelles was also observed (1992). Likewise, Kernen et al. (1997) observed that thylakoid membranes entrapped in reverse micelles formed with CTAB exhibit substantial ATPase activity. Furthermore, Hoechkoeppler and Luisi (1991) observed oxygen uptake by carrot cells in a system formed with AOT and isooctane.

However, Pfammater et al. (1992) made some remarkable observations. One of the most complex functions of living organisms is reproduction; thus, it is fascinating that Pfammater and co-workers observed that the number of yeast cells increased in a system that was formed with Tween 85 in isopropanol.

XIII. CONCLUSIONS

Perhaps the most outstanding finding in systems of low water contents is that enzymes can work in conditions that are far from those that exist in living organisms. However, when one goes to these unnatural conditions, it is remarkable to find that water, albeit in minute quantities, is needed for enzyme function. In retrospect, this may not be surprising because enzymes evolved in a water milieu, and thus water must have been a strong component of enzyme evolution. However, the work in nonconventional systems has opened new panoramas in enzymology. For example, because enzymes can work in essentially nonaqueous media, it now appears feasible to manipulate the equilibrium constant of enzyme-catalyzed reactions by rational adjustments in the solvation energy of the substrates and products.

A second salient feature of the studies of enzymes at low water concentrations is that they have shown that enzymes are quite versatile, that is, they may work at surprisingly high temperatures, express superactivity, and catalyze reactions that had not been seen in water. In this regard, it is pointed out that for probing a particular characteristics of enzymes, each of the two systems that have been discussed here seem to offer advantages over the other. For example, for following the acquisition of the catalytically active three-dimensional structure of proteins in "slow motion", the system of reverse micelles seems quite attractive; fur-

thermore, reverse micelles offer the possibility of studying oligomeric proteins at different levels of association. Reverse micelles may also be used as membrane models, and thus they can be used to gain insight into how the structure of a model protein is affected by its interaction with the surface of an amphiphile. On the other hand, for producing changes in substrate specificity, or for the freezing of enzyme conformations that carry out reactions that are not observed in water, enzymes dispersed in organic solvents would seem to be the system of choice.

There are still many questions to be answered on enzymes in low water environments. A particular relevant point concerns the structure that enzymes acquire in such conditions; important work has been done, but further information is needed. Nonetheless, the studies that have been carried out in the last 15 years have shown that enzymes have many unsuspected properties; this by itself is a major contribution of the field of noncoventional enzymology and suggests that further studies will perhaps reveal other unsuspected properties of enzymes.

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