Protein Dynamical Structure by Tryptophan Phosphorescence and Enzymatic Activity in Reverse Micelles: 2. Alkaline Phosphatase

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Alkaline phosphatase from *E. coli* was solubilized in reverse micelles of bis(2-ethylhexyl) sodium sulfosuccinate in isooctane. The catalytic rate constant in this solvent mixture has a characteristic bell-shape dependence on the water content (w_0) of the micelles and under optimal conditions is 5–6 times larger than in aqueous solution. The dynamical features of the macromolecule, as revealed by the phosphorescence lifetime of Trp-109, are strongly influenced at small value of w_0 and show that activation of the catalytic functions between $w_0 = 20$ and $w_0 = 31$ is accompanied by a reduction in flexibility of the macromolecule in the region backing up the active-site cavity. On the other hand, the decrease in the catalytic rate constant with micelle of large radii is not followed by detectable changes in the enzyme's conformation, suggesting that a lower turnover number probably reflects the altered reactivity of micellar water, a finding that could by itself account for the phenomenon of superactivity.

Introduction

Alkaline phosphatase, AP, from *E. coli* is a dimeric enzyme that catalyzes the hydrolysis of phosphate monoesters. Of the three tryptophan residues per subunit, Trp-109 is surrounded by a β-plaited sheet and α-helical rods, a region of the macromolecule that acts as a support to the active-site pocket.[1] Thanks to the extraordinary rigidity of this structural agglomerate, Trp-109 has the ability to phosphoresce at room temperature in aqueous solution.[2,3]

In a previous study[4] the sensitivity of the triplet-state lifetime of the indole nucleus to the local viscosity[5] was shown to provide a useful tool in probing the dynamical structure of alcohol dehydrogenase in reverse micelles of AOT. With AP we may extend this approach to yet another enzyme and test whether the relationship found between enzyme structure and catalytic efficiency in the micellar state is of a general significance.

Alkaline phosphatase is a particularly stable macromolecule in solution, and drastic conditions are required to affect its structure (phosphorescence lifetimes) and/or catalytic efficiency. Perturbations in its structure due to the micellar state would confirm that confinement in the water pool imposes considerable stress on the native conformation. Hydrolyzing enzymes, in addition to displaying a bell-shape dependence of the catalytic rate constant on the water content of the reverse micelle, often show a much greater efficiency in this solvent system than in buffer (superactivity).[6-9] With acid phosphatase, for example, the turnover number may be enhanced by over 200 times.[10] Should this characteristic be confirmed also with AP, we may able to distinguish whether and to what extent the phenomenon of superactivity is to be attributed to a particularly favorable conformation of the macromolecule or to other circumstances.

We report here that AP introduced in reverse micelles of AOT possesses a catalytic rate constant having both a bell-shape dependence on the water content and an optimal activity a few times larger than in aqueous solution. The kinetics of the phosphorescence emission reveal important perturbations in the structure of the macromolecule and show that activation of the catalytic function is accompanied by a reduction in flexibility of the region about Trp-109. They also suggest that the decrease in turnover number with the larger micelles and the phenomenon of superactivity are due to the peculiar properties of micellar water.

Materials and Method

Electrophoretically purified alkaline phosphatase, AP, type III-R from *E. coli*, and bis(2-ethylhexyl) sodium sulfosuccinate (AOT) were obtained from Sigma Chemical Co. (St. Louis). AOT was purified according to Wong et al.[11] and dried in vacuo over P_2O_5. The purity of AOT was checked by the procedure of Luisi et al.[12] Spectroscopic grade isooctane and glycerol from Merck (Darmstadt) were used without further purification. The enzyme was dialyzed for at least 24 h under nitrogen against Tris–HCl 0.1 M buffer, pH 8.0. Any remaining insoluble precipitate was removed by centrifugation.

Micellar solutions of desired w_0 were obtained by adding, with a microsyringe, concentrated protein in Tris–HCl 0.1 M, pH 8.0, to a 100 mM AOT solution in isooctane. The protein concentration, evaluated by using the extinction coefficient in water, was kept constant to a value of 5.4×10^{-6} M. Stable micelles, after introduction of AP, were obtained provided that the ionic strength of stock Tris buffer solution did not exceed 0.1 M. Their stability at low temperature is rather limited for the large w_0, clear solutions being observed only above 20 °C. All experiments reported in this study were carried out at 25 °C.

The activity of AP in water was measured by the direct spectrophotometric measurement of the hydrolysis of *p*-nitrophenyl phosphate according to ref 13. Since the product of this reaction, *p*-nitrophenol, was shown to interact with the AOT surfactant,[14] thereby changing its absorption spectrum, the following procedure was adopted for activity measurements in reverse micelles. Micellar solutions of the same w_0, one containing the enzyme (5×10^{-10} M) and the other the substrate, *p*-nitrophenyl phosphate, were mixed, and aliquots of 1 mL were withdrawn at regular time intervals. To each aliquot, 1 mL of 1 M NaOH at about 100 °C was added to stop the reaction, and the concentration of *p*-nitrophenol partition in the water phase was determined spectrophotometrically. From independent experiments with mixtures of *p*-nitrophenolate and *p*-nitrophenyl phosphate we have deter-

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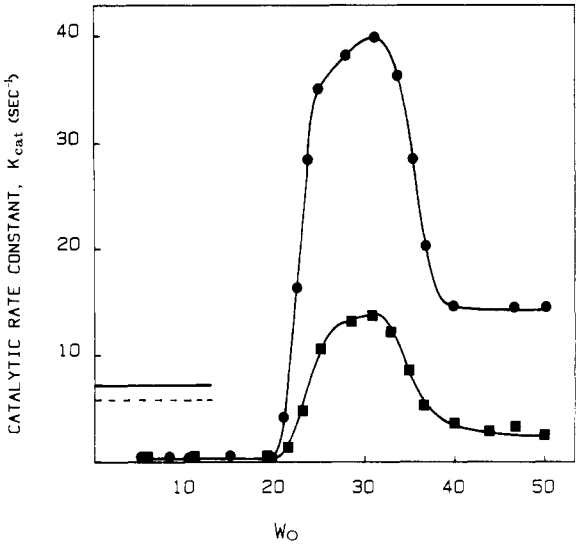

Figure 1. Turnover number (k_{cat}) of AP in micellar solutions as a function of w_0 at pH_{st} 9.0 (■) and 8.0 (●). The enzyme concentration (overall) was 1.5×10^{-9} M. The straight lines represent the value of k_{cat} in 0.1 M Tris–HCl buffer at pH 8.0 (—) and 9.0 (- - -).

mined that the partition coefficient in water was about 0.97 and that no additional hydrolysis of the substrate occurs during this treatment.

Sample Preparation for Luminescence Measurements. It is of paramount importance to remove thoroughly all dissolved oxygen to obtain reproducible phosphorescence data in fluid solutions. Satisfactory deoxygenation of the sample was achieved by replacing air with very pure nitrogen by a procedure previously described.[15]

Various O_2 and NO concentration at a given temperature were introduced by equilibrating the thermostated solution for about 15 min with known partial pressures of O_2 and NO. Partial pressures were determined from the overhead pressure (digital pressure meter OG 713 and OG 973, Officine Galileo, Florence) and the composition of appositely prepared mixtures of these gases with N_2 (SIO, Florence). The same results were obtained by either varying the overall pressure of a given gas mixture or maintaining the pressure constant and changing the mixture composition. Final concentrations of O_2 and NO were calculated by using Henry's law and the solubility of these gases in water (*Handbook of Chemistry and Physics, 41st ed.*). Enzyme activity measurement before and after degassing showed no alteration in the sample.

Spectroscopic Measurements. Fluorescence and phosphorescence spectra were obtained with a conventionally designed instrument.[15] The excitation was selected by a 250-mm grating monochromator (Jarrel-Ash) employing a band-pass of 2 nm for fluorescence and 10 nm for phosphorescence. The emission was dispersed by a 250-mm monochromator (Jobin-Yvon H25) and detected with an EMI 9635 QB photomultiplier. Variations in the lamp output were accounted for by normalizing fluorescence and phosphorescence intensities with the intensity of the exciting light.

Phosphorescence decays were monitored at 440 nm by a double shutter arrangement, permitting the emission to be detected 2 ms after the excitation cutoff. The decaying signal was stored, on occurrence averaged in a Varian C-1024 time-averaging computer, and successively transferred to an Apple II computer for exponential decay analysis by a least-squares method.

Fluorescence anisotropy measurements were carried out by inserting linear polarizers, Polaroid type HNP, B, in both the excitation and emission beam. The excitation wavelength was 300 nm, while the emission was centered at 330 nm.

Circular dichroism spectra were obtained with a Jasco Model J/500 A recording spectropolarimeter utilizing a cell of 1-cm path length.

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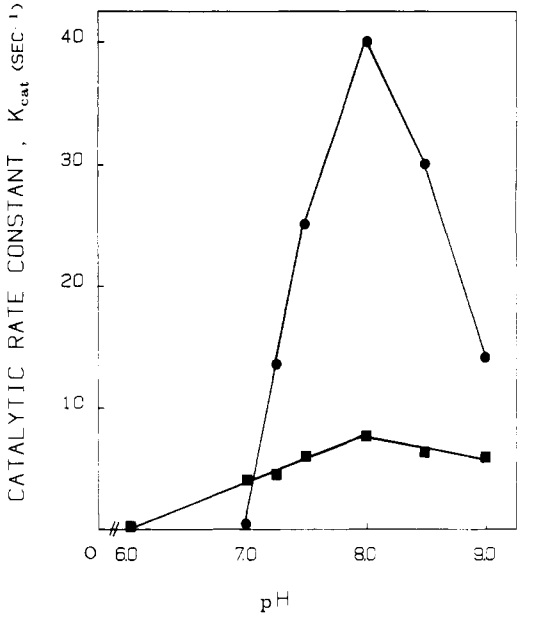

Figure 2. Turnover number (k_{cat}) of AP as a function of pH in micellar solution of w_0 = 31.1 (●) and in Tris–HCl 0.1 M buffer (■). The enzyme concentration was 1.5×10^9 M.

Results

Enzymatic Activity of AP in Reverse Micelles. The activity of AP solubilized in reverse micelles of AOT in isooctane was found, as for many other enzymes, to depend rather drastically on the water content of these structures. The data reported in Figure 1 for the variation of the catalytic rate constant, k_{cat}, as a function of w_0 at two different pHs show a characteristic bell shape and a maximum activity that at pH 8 is 5.3 times larger than the corresponding value in buffer. At both pH 8 and 9 onset of enzyme activity occurs around w_0 = 20, and k_{cat} rises suddenly to its maximum value at w_0 = 31.5. From the correspondence between w_0 and micellar size (ref 16–18) we note that this enzyme becomes functional when the radius, R, of the water core is $R \geq 38$ Å, the highest turnover being reached when R = 60 Å. As the hydrated radius of AP (M_w = 88 000) may be estimated to be about 36 Å, catalytic activity is detectable when the dimensions of the water pool in empty micelles are comparable to those of the macromolecule. For maximum activity the volume of the water pool is about 4 times larger. This finding departs from what seems to be the tendency with some other enzymes that maximum activity is to be observed when the water pool of micelles is roughly of the same size as the macromolecule.[9]

Figure 2 reports k_{cat} as a function of the pH of the stock buffer for micelles of w_0 = 31.5 and compares it with the pH profile in aqueous solution. Maximum activity is found around pH 8 both in solution and in the micellar phase. The major difference in the pH profiles is represented by a narrow range over which the enzyme functions in the micellar milieu. Incidentally, the correspondence in the pH of maximum activity and the symmetry observed in Figure 1 for k_{cat} at different pHs are both indications that under the present experimental conditions the pH of the water pool does not depart from that of the stock buffer.

Tryptophan Steady-State Emission Properties. AP possesses three tryptophan residues per subunit. The fluorescence and phosphorescence spectra of the enzyme incorporated into reverse micelles have at all w_0 the same wavelength for the maxima and the same width of vibronic bands as in aqueous solutions. Any perturbation that might occur in the conformational state of the macromolecule does not then affect the chemical nature of the microenvironment of these chromophores. Similarly, the

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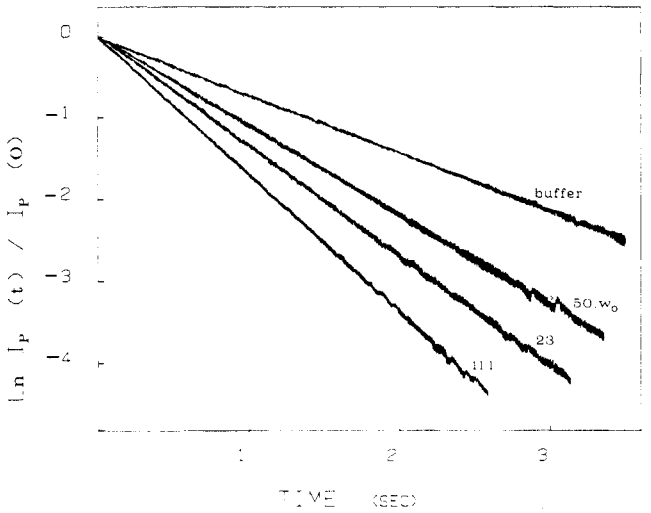

Figure 3. Decay of the phosphorescence intensity of AP in Tris–HCl 0.1 M pH 8 and in micellar solutions at some representative w_0 (λ_{ex} = 300 nm, λ_{em} = 440 nm).

steady-state fluorescence anisotropy, r, in micelles (λ_{ex} = 300 nm, λ_{em} = 330 nm) is constant across the entire range of w_0 with a value of $r = 0.165 \pm 0.003$, practically identical with that observed in aqueous solution. This suggests that processes such as dissociation of dimers into monomers, independent rotational motions of indole side-chains on a nanosecond time scale, and energy migration among chromophores are to a large extent unaffected. The same conclusion regarding the overall structure of the protein is reached upon investigation of the secondary–tertiary structure with circular dichroism. The CD spectrum and the ellipticity obtained in buffer is the same as with micelles of various w_0.

Kinetics of the Phosphorescence Emission. The decay of the phosphorescence intensity with time at a few representative w_0 and in buffer is shown in Figure 3. These data refer to micellar concentrations corresponding to 100 mM in AOT. Variations in micellar concentration from 20 to 200 mM and in the fraction of micelles filled with protein did not affect the decay. Thus, second-order processes arising from collisions with either filled or empty micelles do not seem to influence the kinetics of the phosphorescence emission.

For all samples studied the decay of the phosphorescence obeys a strictly monoexponential law. Since this is possible only if all phosphorescing residues are in a microenvironment of similar flexibility, an exponential decay implies that these dynamical properties are the same in all emitting proteins. In Figure 4 we report the lifetime-normalized phosphorescence intensity, I_p/τ, a parameter that is directly related to the fraction of macromolecules contributing to the emission. The observations that I_p/τ is invariant to w_0 and possesses the same value as in buffer together with the detection of a single phosphorescence lifetime imply that the protein sample is homogeneous throughout. Therefore, partial denaturation and partition of the biopolymer in different microphases of the solvent are ruled out.

Data on lifetime and k_{cat} are compared in Figure 4 for w_0 ranging from 10 to 50. Below $w_0 = 8$ the enzyme activity is hardly measurable, the phosphorescence is completely quenched, and the fluorescence spectrum is blue shifted by 10–15 nm. All these indications denote that the enzyme must undergo profound alteration in structure if not outright denaturation. For $w_0 \geq 10$ the phosphorescence lifetime of AP is always smaller than in buffer (τ = 1.6 s), the departure being more pronounced in the range of w_0 from 10 to 20. This smaller value of 0.65 ± 0.02 s increases to a plateau of 1.00 ± 0.05 s at larger w_0, the change occurring in correspondence with the full restoration of the catalytic function. The shorter τ of Trp-109 in micelles attests to a greater flexibility of the β-plaited sheet bordering the active-site region. This may be quantified in a decrease of local viscosity by a factor of 2.6–6.[5] Like for liver alcohol dehydrogenase,[4] this loosening of the internal structure is not imputable to bulk matrix effects such as the

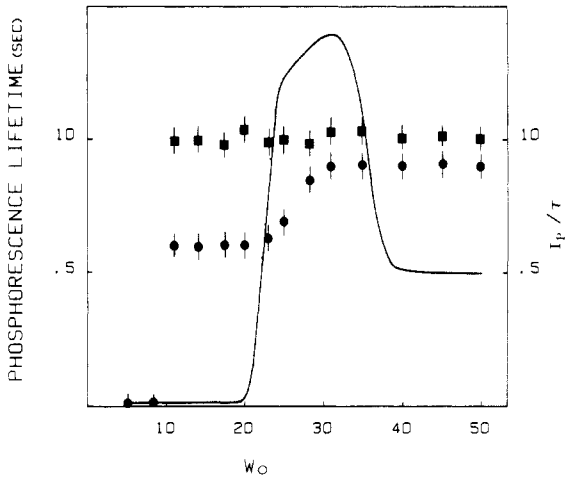

Figure 4. Dependence of the triplet-state lifetime, (●) of Trp-109 of AP on the w_0 of the micellar solution compared to the catalytic activity. Lifetime-normalized phosphorescence intensities, I_p/τ (■) relative to the value of this parameter in buffer are also included.

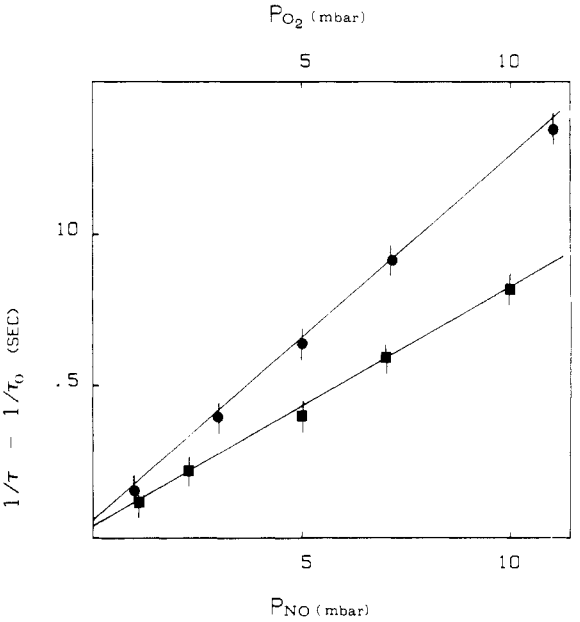

Figure 5. Dependence of the phosphorescence lifetime of AP in reverse micelles at w_0 = 24.4 on the partial pressure of O_2 (●) and NO (■). Errors bars represent the range in the values obtained with three independent experiments.

viscosity of the water pool. Indeed, τ is not affected by water/glycerol solutions 100 times more viscous than water. The greater flexibility of the protein structure probably signals changes in conformation of the macromolecule in response to specific interactions with the surfactant or the organic solvent.

Quenching of the Phosphorescence by Oxygen and Nitric Oxide. Quenching of the phosphorescence by O_2 and NO requires that these molecules diffuse to the inner folds of the biopolymer. Because this process is largely modulated by fluctuations in protein structure, the magnitude of the second-order quenching rate constant is a measure of its flexibility. The decay of the phosphorescence in the presence of O_2 and NO is monoexponential, and the dependence of τ on the partial pressure of these gases is displayed in Figure 5. For every w_0 investigated the quenching rate, $1/\tau - 1/\tau_0$, is proportional to the partial pressure of the quenchers, thus confirming that quenching is a purely dynamic process. The rate of quenching may be expressed in the usual way as $k_Q[Q]$ where k_Q is the second-order rate constant and [Q] is the appropriate concentration of quencher in solution.[19] Ac-

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cording to the work of Gandin,[20] the concentration of O_2 in the water pool of AOT micelles in isooctane is not dissimilar to that in bulk water, being only about 25% smaller. Since oxygen-transfer rates in and out of the water core are rapid in comparison to the quenching process ($k_{in} = 8.9 \times 10^6$ s^{-1}, $k_{out} = 7.8 \times 10^7$ s^{-1}, $k_{O_2} \simeq 10^6$ M^{-1} s^{-1}),[15] we may assume that the quencher is in equilibrium between the two microphases and that the appropriate O_2 concentration is given by the solubility of this gas in bulk water. That this is a reasonable approximation is demonstrated by the value derived for k_{O_2} and k_{NO} ($k_{O_2} = 1.3 \times 10^6$ M^{-1} s^{-1}, $k_{NO} = 8.9 \times 10^5$ M^{-1} s^{-1}), molecules that in solution were shown to have analogous permeabilities for AP ($k_{O_2} = 1.5 \times 10^6$ M^{-1} s^{-1}, $k_{NO} = 9.1 \times 10^5$ M^{-1} s^{-1}).[3] On the other hand, were the quenching reaction limited by the rate of transfer of O_2 across the micellar wall (expected to be similar for the two molecules and be proportional to the concentration of these gases in isooctane), the preferential solubility of O_2 in isooctane as compared to NO[22] would yield k_{O_2} to be about 3 times larger than k_{NO}. The rate constants k_{O_2} and k_{NO} are unaffected by the water content of micelles and within the experimental uncertainty have the same magnitude as in aqueous solution. Because the quenching process samples mostly the flexibility of an outer shell of the macromolecule,[3] rather than the inner core about Trp-109, the permeability of AP to O_2 and NO in the micellar state excludes the possibility that severe perturbations occur in the outer layers of the protein molecule.

Discussion

AP from *E. coli* is among those enzymes that in AOT micelles displays both a bell-shape dependence of the catalytic rate on the size of the water pool and a substantial enhancement of the reaction velocity with respect to the aqueous solutions. This last observation is apparently in contrast with a previous report on AP from calf intestine that instead showed a reduced activity in micelles and a shift in optimal pH of 1.5 units.[23] In light of the present results, it is evident that the previous investigation was carried out in an unfavorable range of w_0 ($w_0 = 10$ and 14.8) for which even AP from *E. coli* is inactivated and shifts in the pH of the water pool are normally the rule.

A fundamental question regarding enzyme kinetics in reverse micelles is to find out whether and to what extent phenomena such as the bell-shape dependence of k_{cat} on w_0 and enzyme superactivity may be associated to peculiar conformational states of the macromolecule.[24] The experimental findings reported here confirm that the structure of AP in H_2O/AOT/isooctane solvent mixture is different from that of the native state in buffer, and particularly in the low range of w_0 its properties are profoundly influenced by the water content of reverse micelles. Below $w_0 = 10$, large-scale structural rearrangements or denaturation are manifested by pronounced changes in the luminescence properties of its tryptophan residues. Above this threshold, at $w_0 > 10$, steady-state spectroscopic measurements such as absorption, fluorescence, and circular dichroism spectra identify the enzyme as in essentially the native conformation. At these w_0s only the phosphorescence lifetime of Trp-109, which reports on the flexibility of the macromolecule not far from the active site, signals important changes in the enzymes. In this regard, it is important to recognize that variations in flexibility of this highly structured region have so far been induced only by drastic treatments and that they are invariably followed by a loss of enzymatic function. AP is resistant to heat denaturation and is impervious to the unfolding action of concentrated urea and guanidine hydrochloride. No denaturation (nor change in phosphorescence lifetime) is observed in 8 M urea. Loss of enzyme activity with guanidine hydrochloride requires concentrations greater than 3 M. Under such conditions of reduced activity the phosphorescence lifetime was found to decrease in a parallel fashion.[25]

In micelles of AOT onset of catalytic function requires that the water pool be of dimensions at least comparable to the hydrated macromolecule. Full activation of the enzyme is attended by an increase in rigidity of the macromolecule approaching more closely the native state. On the other hand, the further drop in catalytic rate, which from $w_0 = 31$ to $w_0 = 50$ reduces to about 40% of the maximum value, is not accompanied by visible changes in the conformation of the macromolecule. Thus if the recovery of the catalytic function is conceivably related to the restoring of productive configurations of the protein molecule, the successive slowing down of the reaction rate as the amount of free water increases does not seem to be attributable to further changes in the protein structure. This observation prompted us to consider that in large micelles the turnover number is possibly dominated by changes in the reactivity of the substrates. Among them, micellar water, a reactant in the hydrolysis of phosphate monoesters, must be singled out for its peculiar nucleophilic properties and activity, which with respect to bulk water may be enhanced by orders of magnitude and may depend strongly on w_0.[26,27]

Should the gradual loss of enzymatic activity in this range of w_0 be correctly ascribed to the decreased reactivity of micellar water as it approaches its bulk properties, then we do not require particularly favorable functional states of the enzyme to account for the phenomenon of superactivity. Perhaps it is not a mere coincidence that supractivity has been found with enzymes such as α-chymotrypsin, peroxidase, and acid phosphatase, to mention the most notable examples, all catalyzing reactions in which water is a reagent.

Finally, by employing an intrinsic probe of protein structure, shielded away from direct interactions with the solvent, we are able to appraise the often presumed important role that the water structure in micelles might have on the dynamical state of the macromolecule. At very small w_0 all water molecules are immobilized in primary hydration shells, and the absence of free water would be expected to show up in a dampening of the natural fluctuations in the protein structure. This phenomenon is normally observed upon dehydrating proteins, a process followed by big reduction in flexibility and severe consequences to catalytic function.[28,29] The results with AP and with liver alcohol dehydrogenase[4] demonstrate that in micelles the scarcity of water instead causes a greater flexibility in the inner core of the macromolecule, thus pointing out that interactions of a nature different from hydration are responsible for altering the native conformation. Moreover, even in abundance of free almost bulklike water, at $w_0 = 50$, both alkaline phosphatase and alcohol dehydrogenase are in a conformation departing significantly from the native one. Such considerations induce us to think that the perturbations on the structure of the macromolecule arise rather from specific interactions with the surfactant (or the organic solvent) than from the structure of micellar water. This could also explain the variability in catalytic efficiency with different surfactants,[7,30] a behavior well established with water-insoluble enzymes in normal micelles.[9]

Registry No. AP, 9001-78-9; AOP, 577-11-7; *p*-nitrophenyl phosphate, 330-13-2.

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