and aliquots of each (0.6 mL) were withdrawn, dissolved in PCS solubilizer (Amersham/Searle), and assayed for radioactivity. Seven areas of radioactivity were observed. The fractions comprising each peak and the percent of total recovered radioactivity in each were as follows: 8–14, 2%; 21–25, 4%; 28–32, 8%; 33–41, 16%; 54–59, 2.5%; 75–82, 21.5%; 83–92, 46%.

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Micellar Solubilization of Biopolymers in Organic Solvents. 5. Activity and Conformation of α-Chymotrypsin in Isooctane–AOT Reverse Micelles

Slobodan Barbaric and Pier Luigi Luisi*

Contribution from the Technisch-Chemisches, Laboratorium der ETH-Zürich, ETH-Zentrum, 8092 Zürich, Switzerland. *Received August 25, 1980*

Abstract: The enzymatic activity of α-chymotrypsin solubilized in reverse micelles formed in isooctane by bis(2-ethylhexyl)sodium sulfosuccinate and water (0.6–2.5% v:v) has been investigated with the use of *N*-glutaryl-L-phenylalanine *p*-nitroanilide as the substrate. The enzyme obeys Michaelis–Menten kinetics in the investigated concentration range, with K_m values which are considerably higher than those in bulk water (when concentrations are referred to as water pools). Under certain conditions, there is an enhanced turnover number (up to a factor of 6) in micelles with respect to the aqueous solution. The pH profile of the enzyme activity in the hydrocarbon micellar solution is different from that in water, being shifted to higher pH values and the more so the lower the water content. This appears to occur via a complex mechanism in which most likely the pK of the enzyme's active groups is modified by the peculiar nature of the solvent in the water pools. Fluorescence studies show only minor changes of the protein emission with respect to aqueous solutions, which is taken as a confirmation of the enzyme confinement within the water pool of the reverse micelle. Circular dichroism studies show significant changes in both the near- and far-UV regions, and the marked intensification of the ellipticity in the 230-nm region at lower water concentration is interpreted as arising from an increase in the content of helical structure. This is attended by a parallel increase of the enzymatic activity. Furthermore, under conditions of low water content (0.6–1% v:v) the enzyme's stability is greater than in aqueous solution. Thus, it appears that in micelles with low water content the enzyme assumes a more rigid conformation and has a higher stability, a higher turnover number, and at the same time a lower kinetic affinity for the substrate. These structure and activity changes of the enzyme are discussed in terms of the size and structure of the micellar aggregate.

It has been shown independently by Martinek et al.,[1] by Menger and Yamada,[2] by Douzou et al.,[3] and by our group[4] that hydrophilic enzymes can be solubilized in hydrocarbon solvents with the help of ionic surfactants and a small amount of water. According to a tentative structural model recently proposed,[4c] the protein is confined in the water pool of the reverse micelles formed by the surfactant molecules, and a layer of water separates and protects the protein surface from the inner surface of the surfactant layer. Such enzymatic systems may be technologically interesting, e.g., for the catalytic transformation of lipophylic substrates. Aside from this practical aspect, a number of questions arise which are of general interest both for the micelle field and for protein chemistry. One such question concerns the relation between the enzyme's reactivity and the enzyme's environment provided by the micellar core. The relevance of this question will be apparent in this paper, as it will be shown that under certain conditions the enzyme in the hydrocarbon micellar solution has a higher turnover number than that in bulk water. Another question of general interest concerns the nature of structural changes induced in the protein upon its confinement in the water pool. We will show here that, in parallel with the case of lysozyme,[4d] marked changes in the main chain's conformation can occur without impairing the enzyme's activity. Finally, one should recognize that the reactivity in a micellar system containing the enzyme and the substrate is based upon the encounter among different micellar species: i.e., there is a mechanism of mass transport across microinterfaces, a problem which is of interest in several areas of chemistry, including membrane chemistry.

At the present state, our knowledge of these micellar aggregates is very limited, and in this paper we address only part of these questions. In particular, we describe the properties of α-chymotrypsin solubilized in isooctane with bis(2-ethylhexyl)sodium sulfosuccinate (AOT), and we will try to correlate the enzymatic activity with the structure of the micellar aggregate and with the conformation of the protein. An analogous paper on the activity and stucture of lysozyme in reverse micelles is presented elsewhere.[4d]

Experimental Section

Materials. Bis(2-ethylhexyl)sodium sulfosuccinate (AOT) was obtained from Serva, purified as previously described[4b] and stored over P_2O_5 in an evacuated desiccator. A 50 mM solution of purified AOT in isooctane had generally an absorbance less than 0.03 at 280 nm.

Isooctane puriss was purchased from Fluka. α-Chymotrypsin was obtained from Boehringer, *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) from Serva and *N*-*trans*-cinnamoylimidazole from Sigma. Deionized water was used throughout.

Preparation of Enzyme and Substrate Micellar Solutions. The enzyme and substrate micellar solutions were prepared by injecting with a mi-

* Dedicated to Professor Piero Pino on the occasion of his 60th birthday.

(1) Martinek, K.; Levashov, A. V.; Klyachko, N. L.; Berenzin, I. V. *Dokl. Akad. Nauk SSSR* **1977**, *236*, 920–923.
(2) Menger, F. M.; Yamada, K. *J. Am. Chem. Soc.* **1979**, *101*, 6731–6734.
(3) Douzou, P.; Keh, E.; Balny, C. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 681–684.
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Table I. Dependence of the Enzymatic Reaction Velocity on the Water Content of Reverse Micelles at Constant w_0[a]

|AOT|, mM	% H_2O, v:v	|E|,[b] μM	substrate, mM: overall	substrate, mM: water pool	v,[c] s^{-1}
25	0.6	1.2	0.2	33.2	1.46
50	1.2	1.2	0.2	16.7	0.99
100	2.4	1.2	0.2	8.3	0.50

[a] This is the α-chymotrypsin-catalyzed hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide at w_0 = 13.5, pH_{st} 9.8. [b] This is the overall concentration. [c] This is the initial velocity divided by the enzyme concentration. This figure is independent of the choice of the concentration coordinates (overall or local), since $(v)_{ov}$ should be divided by E_{ov}, and $(v)_{wp}$ (which is $(v)_{ov}/E_w$) should be divided by E_{wp}.

crosyringe the buffered enzyme and substrate stock solutions into the isooctane–AOT solution (50 mM unless otherwise specified). A 0.1 M glycine NaOH buffer was used throughout. The desired water content was obtained by an additional injection of the same buffer solution into the enzyme and substrate micellar solutions, which were then shaken mildly until clear solutions (no scattering at 320 nm) were obtained.

Clear micellar solutions of the enzyme could only be obtained under a restricted range of conditions, depending upon the water content, the pH_{st},[5] the enzyme concentration, and temperature. In the range of enzyme and AOT concentrations investigated in this work, the viable w_0 (w_0 = [H_2O]/[AOT]) range is for example: 9–28 (which corresponds to 0.8–2.5% water v:v) at pH_{st} 9.2 and w_0 = 5.5–17 at pH_{st} 11.8. In other words, the operational w_0 range decreases by increasing the pH of the stock enzyme solution. The enzyme concentration used in the various experiments is specified in the figure legends (in overall concentration). Enzyme solutions at low w_0, used for spectroscopic studies (where a higher enzyme concentration is needed), were generally obtained by diluting an enzyme micellar solution previously prepared at higher w_0 with an AOT–isooctane solution. The enzyme concentration was determined spectroscopically on the basis of an extinction coefficient of 50 000 cm^{-1} M^{-1} at 282 nm. The water present in the AOT–isooctane solutions (because of the hydroscopic nature of the surfactant) was found to be negligible by NMR spectroscopy (less than 0.05% v:v). No activity in a water solution of α-chymotrypsin saturated with AOT (<50 mM) was found.

Enzyme Activity Measurements. The enzyme activity was measured at 30 °C by utilizing *N*-glutaryl-L-phenylalanine *p*-nitroanilide as substrate.[6a] The reaction was carried out as follows: both the sample and 1-ml reference cells were filled with 0.9 mL of AOT–isooctane solution containing substrate at a certain w_0. To the reference cell was added 0.1 mL of the same AOT hydrocarbon solution but without substrate. The enzyme reaction was initiated by adding to the sample cuvette 0.1 mL of enzyme micellar solution, prepared at the same w_0 as the substrate solution. Absorbance of released *p*-nitroaniline was monitored for ca. 10 min (under our conditions, the time progress of the reaction was found to be always linear in such a time interval). In micellar solutions, the maximum of *p*-nitroaniline absorbance was found at 360 nm in contrast to water solution where the maximum is at 380 nm. The reaction was followed at 386 nm for water and 366 nm for micelles, where the absorbance due to the *p*-nitroanilide is negligible. The same molar extinction coefficient of 13 000 M^{-1} cm^{-1} (at 386 nm for water and 366 nm for micelles) was used in both cases. This was calculated from the data of Erlanger et al.[6b] The autohydrolysis of the substrate, once injected into the micellar solution, was negligible compared with the enzymatic reaction even when water stock solutions of the substrate at high pH were used (the substrate was anyway present in the reference cell).

Enzyme preparations used in our study were titrated with *N*-*trans*-cinnamylimidazole in water, pH 7 according to the established procedures,[7] and about 83% of the enzyme was found to be active. The data of Tables I and II have been corrected for this, but not the data of Figures 1, 2, and 5. All activity and spectroscopic measurements were carried out at 30 °C because of the better solubility of the enzyme and the substrate in micellar solutions. This allowed us to investigate a much wider range of w_0. Other experimental data are given in the legends for the figures.

Table II. Kinetic Parameters[a] for the α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Glutaryl-L-phenylalanine *p*-Nitroanilide in Water and in AOT-Reverse Micelles[b]

conditns	$K_{m,app}$, mM	$10^2 k_{cat}$, s^{-1}
water solutions (pH 7.9)	0.6	0.84
reverse micelle, w_0 = 13.5 (pH_{st} 9.8)	38[c] (0.45)	2.71[c,d]
reverse micelle, w_0 = 9 (pH_{st} 11.8)	50[c] (0.40)	5.06[c,d]

[a] Determined by Lineweaver–Burk plots, in the linear concentration range (overall) of substrate 0.05–0.4 mM. The experimental uncertainty of K_m and k_{cat} in the micellar solution is ca. ±20%. [b] 50 mM AOT. [c] The first figure is $(K_m)_{wp}$ and the second, $(K_m)_{ov}$. [d] See footnote *c* of Table I.

Spectroscopic Measurements. Absorbance measurements were carried out with an Acta MVI (Beckman), fluorescence with an Aminco 1000 (a corrected instrument), and circular dichroism measurements with a Jasco J40.

Results and Discussion

Enzyme Activity in the Micellar Hydrocarbon Solution. Before considering the results, it is necessary to recall that in a hydrocarbon micellar solution, concentrations (and therefore K_m) can be expressed in two different ways.[4c] One is relative to the water pool, where reaction takes place and the other to the overall system (water and hydrocarbon). For a compound which is practically insoluble in the hydrocarbon solvent, like an enzyme or our substrate, the two concentrations are correlated simply by eq 1,

$$C_{ov} = C_{wp}F_w$$

where F_w is the percent water and C_{ov} and C_{wp} are the overall concentration and the local (water pool) concentration. It appears to us that C_{wp} is the physically significant one. A simple experiment which shows that this is so is illustrated in Table I. When w_0 and the overall enzyme and substrate concentration are kept constant, the velocity increases by decreasing the water content in the system. This can be explained by a change in the "local" concentration of the substrate in the water pool, which affects the velocity since its concentration is not as a saturating level.[8] Note that the velocity is not affected by the choice of the the system (water pool or overall) once it is normalized for the respective enzyme concentration.[4c]

Let us consider now the enzyme behavior in a wide range of pH and w_0. This preliminary screening was necessary in order to find the conditions of optimal enzyme activity in the hydrocarbon micellar solution. It was not possible to carry out these studies at saturating concentration of the substrate, and therefore they have been all performed at the maximal concentration which could be reached at the lowest w_0 investigated. Figure 1 reports the reaction rate as a function of w_0 at different ph_{st}[5]. For each pH_{st} a bell-shaped curve is obtained, the w_0 optimum ($w_{0,opt}$) shifting toward the left (i.e., to smaller w_0 values) with increasing pH_{st}. Notice that the activity curves are narrower for more alkaline pHs,[9] that the enzyme activity increases at lower $w_{0,opt}$ values, and that all activity curves, regardless of pH_{st}, begin at the minimal w_0 = 6.6. The bell-shaped activity curves reflected in Figure 1 have also been found for other enzymes, for example, for horse liver alcohol dehydrogenase-catalyzed reduction of ac-

(5) In this and other papers of our group, pH_{st} is the pH of the stock aqueous solution injected into the hydrocarbon–AOT solution. The local pH (which is not necessarily the same as pH_{st}) is called pH_{wp}. See ref 4e for more details.

(6) (a) Erlanger, B. F.; Edel, F.; Cooper, A. G. *Arch. Biochem. Biophys.* **1966**, *115*, 106–210. (b) Erlanger, B. F.; Kokowsky, N.; Cohen, W. *Ibid.* **1961**, *95*, 271–278.

(7) Schonbaum, G. R.; Zerner, B.; Bender, M. L. *J. Biol. Chem.* **1961**, *236*, 2930–2935.

(8) The possibility that the different velocities are due to an effect of the different AOT concentrations on the enzyme has been checked by CD measurements. No effect of AOT concentration on the enzyme's ellipticity was observed.

(9) It was also possible to rule out that the behavior represented by the bell-shaped curve was due to an irreversible enzyme inactivation at w_0 values lower and/or higher than $w_{0,opt}$. In order to show this, we prepared micellar enzyme solutions at various w_0s (in the range of 6.6–22.5, pH_{st} 9.8) and then rapidly mixed them with an excess of a micellar solution of the substrate, so that the final reaction mixture had always the same w_0 (13.5) (w_0 optimal for pH_{st} 9.8). The reaction velocity was found to be the same in all cases.

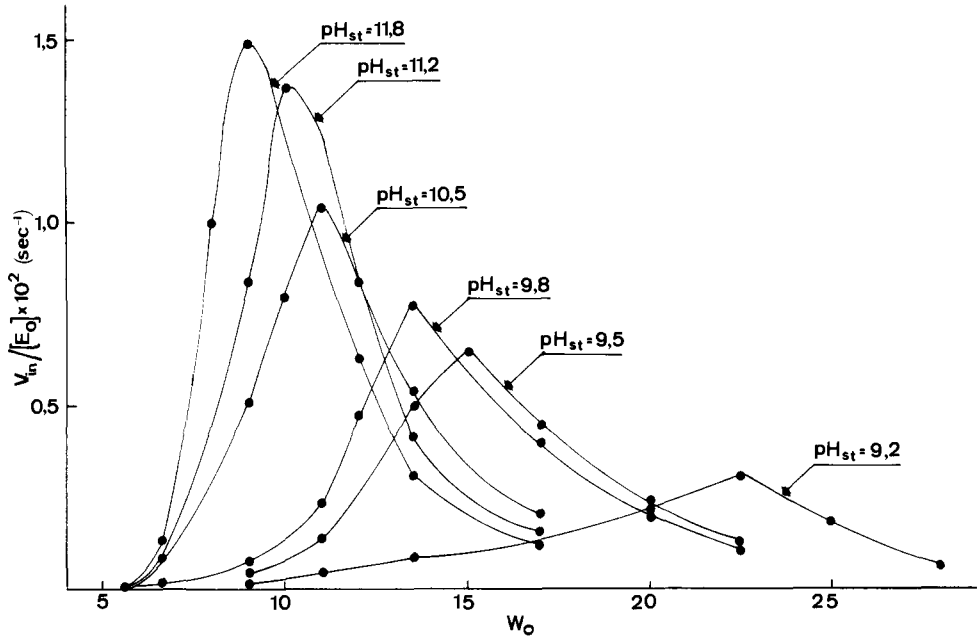

Figure 1. Velocity of the α-chymotrypsin-catalyzed hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide in reverse micelles (50 mM AOT in isooctane) as a function of w_0, at different pH_{st}. $|E|_{ov} = 1.1 \times 10^{-6}$ M; $|S|_{ov} = 2 \times 10^{-4}$ M. For each w_0, the local concentration can be calculated on the basis of eq 1, keeping in mind that $F_w = w_0|AOT|1.8$.

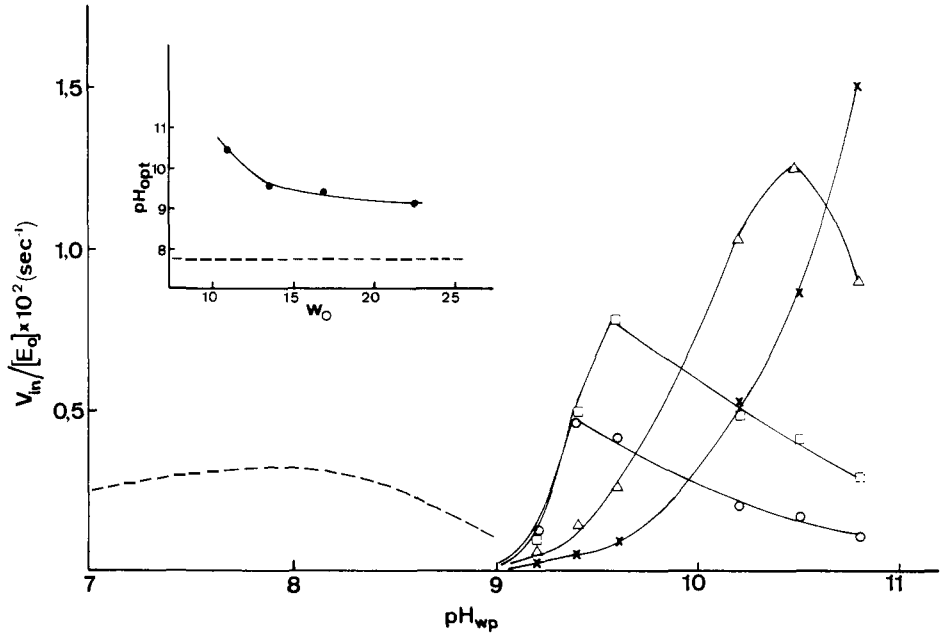

Figure 2. Velocity of the α-chymotrypsin-catalyzed hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide in reverse micelles (50 mM AOT in isooctane) as a function of pH_{wp}, at different w_0: ▲, $w_0 = 9$; ▼, $w_0 = 11$; ■, $w_0 = 13.5$; ●, $w_0 = 17$. The pH_{wp} is obtained by correction of the pH_{st} according to the method described elsewhere.[4c] The broken line represents bulk water at the same overall substrate concentration. Insert: Dependence of pH optimum in the reverse micelles on w_0 (the pH optimum at $w_0 = 22.5$ was determined separately).

etaldehyde[4f] and for lysozyme.[4d] Thus, from these few cases, it appears that the bell-shaped dependence of activity upon w_0 represents a general feature of the micellar enzyme solutions. From Figure 1 it is also apparent that the enzyme displays a higher activity at smaller w_0 values. Partly, this could reflect the effect of substrate accumulation, as discussed on the basis of Table I. However it is clear that this explanation does not account for the whole effect. In fact, the effect on velocity due to substrate accumulation of (for example) the data for $w_{0,opt} = 9$ (pH_{st} 11.8) and the for $w_{0,opt} = 23$ (pH_{st} 9.2) can at the most, be only a factor of 2.5, whereas the data of Figure 1 show a much larger effect. Also differences in K_m alone should not be responsible for this effect, since they appear to be small, as it will be shown later on (Table II). In order to eliminate the possible influence of pH from this kind of comparison, we have replotted data as shown in Figure 2, i.e., velocity vs. pH for the various w_0 values. Notice that pH_{st} has been substituted for the "local" pH, which we call pH_{wp}. Differences between pH_{st} and pH_{wp} can be assessed on the basis of ^{31}P NMR, as described in detail elsewhere.[4e] In our case, corrections were between 0 and 1.0 pH unit (these two extremes were for pH_{st} 9.2 and 11.8, respectively). Also from Figure 2 it is clear that the differences in velocity for the different pH_{opt} are considerably larger than the expected trivial local concentration effects.

Other significant features of the enzymatic reaction in the micellar solution are apparent from Figure 2. One is that the reaction rate is greater in the micellar solution than in water; the other is that the pH_{opt} (which for this substrate in aqueous solution[6a] is 7.8) is shifted remarkably toward more alkaline pH values: the smaller the w_0, the higher is the pH_{opt}. In the insert

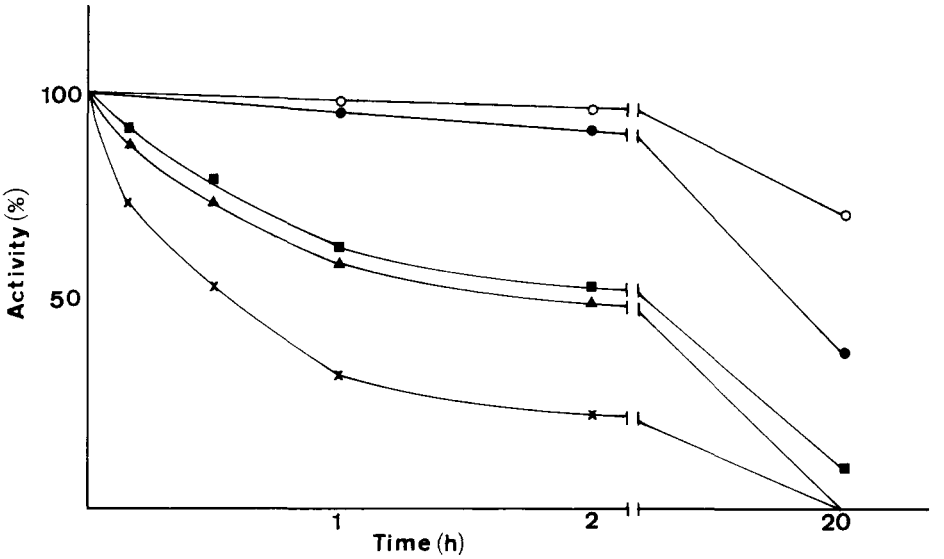

Figure 3. Stability of α-chymotrypsin in water and 50 mM AOT–micellar solutions at different w_0. Enzyme solutions were incubated at 30 °C. Aliquots were taken at the indicated time, and the residual activity was measured as described in the Experimental Section: ○, w_0 = 6.6; ●, w_0 = 13.5; ■, w_0 = 18; ▼, w_0 = 22.5; ▲, water, pH 7.9. $|E_o|$ = 1.1 μM (overall).

of Figure 2, it is shown that the pH_{opt} in micelles changes more markedly in the region of low water content and tends to level off at higher w_0 values. One possible interpretation is that the water content in the micelles directly affects the physical state of the enzyme and brings about an increase of the pK of certain groups in the active site and the more so the lower the water content.

Menger and Yamada, in their recent investigation of the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester in reverse micelles, have also found a marked shift of the pH_{opt}, attended by an increase of the enzyme turnover number. Their interpretation of the changed pH profile is that the enzyme's activity arises from a shift of the pK of the hystidine group in the enzyme-active site.[2]

The observation that the enzyme activity is higher at lower water contents is surprising, particularly if one considers that α-chymotrypsin is a hydrolase and that at low w_0 values most of the water should be bound.[10,11] An independent experiment confirms the higher activity at lower contents of free water: when activity measurements as in Figure 1, pH_{st} 9.8, are carried out with buffer containing 0.2 M NaCl (otherwise the same conditions), greater enzymatic activities and a shift of the $w_{0,opt}$ toward the left are obtained (the bell-shaped curve observed with pH_{st} 9.8 of Figure 1 is practically transformed into the curve obtained with pH_{st} 11.8). The simplest way to explain this effect is to attribute it to the avidity of NaCl toward water.[12] In other words, the salt would tightly bind water in the water pool, thus decreasing the amount of free water. This would result in an increased pK and an increased turnover number.

We have investigated the dependence of the velocity on the substrate concentration at two w_0 values, and the results are given in Table II. In the concentration range investigated, the enzymatic hydrolysis of the substrate follows Michaelis–Menten kinetics.[13] Despite the large experimental uncertainty for K_m and k_{cat}, we can use them to make a few reliable observations. Note for example the significant increase of k_{cat} in reverse micelles with respect to that in water, which was already forseen on the basis of the data of Figures 1 and 2 for unsaturated solutions. Also notice the large increase of $(K_m)_{wp}$ over $(K_m)_{water}$. As was found previously for other enzymes, e.g., horse liver alcohol dehydrogenase[4f] and lysozyme,[4d] the $(K_m)_{ov}$ value is much closer to the value for water. We are tempted to believe that this is accidental and that no clear physical meaning should be attributed to $(K_m)_{ov}$.

If one wishes to measure enzyme efficiency in terms of k_{cat}/K_m, enzyme in the water pool, including α-chymotrypsin under the best conditions, should be considered to be less efficient. But if one considers rates for the same degree of enzyme saturation (e.g., in terms of turnover numbers), α-chymotrypsin under optimal conditions results more active (by at least a factor of 6) in the hydrocarbon micellar phase. Thus the kinetic behavior of enzymes in AOT–reverse micelles seems to follow a peculiar pattern: a rather unfavorable $(K_m)_{wp}$ is attended by a good turnover number.

One problem which also deserves some consideration is the time stability of α-chymotrypsin in the hydrocarbon micellar phase. The activity of proteases in water solution rapidly falls off, mostly due to autolysis. As apparent from Figure 3, the relative activity of α-chymotrypsin in water at 30 °C drops to ca. 50% after 2 h. The decay in the micellar enzyme solution proceeds even more rapidly with w_0 = 22.5, while at w_0 = 18 about the same decay rate as in water is observed. By contrast, at lower w_0 values, the time stability is much better, and at w_0 = 6.6 the protein has still about 70% of its original activity after 20 h. Thus, the micellar conditions which bring about the highest activity also induce an increase stability with time. Most likely, it is possible to further optimize the stability of α-chymotrypsin, e.g., by the addition of $CaCl_2$ or by decreasing temperature.

Spectroscopic Studies. First, it may be useful to recall the structure of the micellar aggregate which hosts the protein, as recently described with a simple model.[4c] In such a model, the dimension of the AOT reverse micelles (assumed to be monodisperse and spherical) depend upon the water content and upon the size of the quest protein molecule. Thus, α-chymotrypsin, which has dimensions 40 × 40 × 51 Å and a molecular volume of ca. 43 000 Å^3,[14] can be represented by a sphere with a radius 22 Å. Our calculations predict that at w_0 = 13.5 (1.2% H_2O), the inner core radius of the protein-containing micelle is ca. 33

(10) Wong, M.; Thomas, J. K.; Nowak, T. *J. Am. Chem. Soc.* **1977**, *99*, 4730–4736.

(11) It has been established that AOT molecules in reverse hydrocarbon micelles may engage up to 6–7 water molecules in the hydration shell.[10] In our case, protein and buffer ions will compete for water, and the final distribution of bound and free water molecules as well as the dynamics of the exchanges from one type of water state to another would be difficult to assess.

(12) The effect of salt addition in relation to the properties of α-chymotrypsin in aqueous solution has been investigated in detail before (Cuppett, C. C.; Resmick, H.; Canady, W. J. *J. Biol. Chem.* **1971**, *246*, 1135–1141). We could also show that addition of KCl brings about an increase of the molar ellipticity at 230 nm.

(13) At higher substrate concentrations (>0.4 mM), we noticed the appearance of inhibition effects, which at this stage have not been further investigated. Martinek et al.[1] also investigated the same substrate in reverse micelles. They also report Michaelis–Menten kinetics, with a K_m similar to that observed for water.

(14) Blow, D. M. "The Enzymes", 3rd ed.; Boyer, P. D., Eds.; Academic Press: New York, London, 1971; Vol. III, pp 185–212.

Table III. Fluorescence Properties of α-Chymotrypsin

conditns	λ_{max}, nm	relative quantum yield	P_{305}[a]
water (pH 7.9) 50% propylene glycol at −70 °C[b]	333.0	100	0.130 0.170
w_0 = 6.6[c]	329.0	113	0.176
w_0 = 9.0	330.0	111	
w_0 = 13.5	330.5	110	0.170
w_0 = 19.0	332.5	100	
w_0 = 22.5	334.5	90	0.088

[a] Degree of polarization at λ_{exc} = 305 nm. [b] Data from Weber.[19] [c] This and the following data refer to an AOT-iso-octane micellar solution, [AOT] = 50 mM.

Å, which means that there is a layer of water of ca. 11-Å thickness which separates the protein surface from the inner layer of surfactant molecules. There will be ca. 4000 water molecules and ca. 290 surfactant molecules per micelle (we assume there is only one protein molecule per micelle).

Let us now consider UV absorption data, as illustrated in Figure 4. Difference spectra of the micellar protein solutions with respect to the protein water solution indicate a perturbation of the aromatic chromophores. This perturbation is generally smaller, the larger the water content. It is known that the aromatic region of the Uv absorption spectrum of α-chymotrypsin is rather sensitive to the environment, and perturbations similar to those reported in Figure 4 have also been obtained for the water solution under certain conditions.[15] Table III reports fluorescence data. Changes of λ_{max} and quantum yield with respect to water are small in the investigated w_0 range (0.6–22.5). In particular, λ_{max} is slightly shifted to the red at w_0 = 22.5 and to the blue at lower w_0 values. At lower w_0 values, the quantum yield is slightly larger than for water solution whereas at w_0 = 22.5 it is slightly smaller than for water solution. Interestingly, the fluorescence properties of the water solution are better matched by a micellar solution with w_0 = 18 and not by the micellar solution with the largest w_0. Table III also reports some data for fluorescence polarization. Polarization of protein fluorescence is generally recognized as a qualitative measure of macromolecular mobility, and those factors which increase the degree of polarization are also assumed to decrease the mobility of the protein.[16] In particular, Weber[17] has investigated α-chymotrypsin in a 50% propylene glycol–water mixture at −70 °C and found an increase of the polarization at λ_{exc} = 305 nm with respect to water. Our data show that the degree of polarization in the micelles changes with respect to that in water in a way which suggests a lower mobility of the fluorophores at lower w_0 values. Remember, from the previous section, that at low w_0 values α-chymotrypsin has a higher activity and a higher stability.

Let us consider now the CD data, which can be more directly related to the conformation of the protein. In this regard, it is useful to recall that α-chymotrypsin is supposed to exist in solution in two major conformational forms, with an equilibrium being regulated by a pK of ca. 8.8 and with the inactive form predominating at a more alkaline pH.[18] The CD band at 230 nm has been ascribed to the helical content of the protein,[19] and the intensity of this band can be correlated to the enzyme activity (for example, it is lower at alkaline pH where the enzyme is inactive and practically absent in the inactive zymogen).[20]

Our data are presented in Figure 5. As a reference, we also give the CD spectra for a water solution at pH 7.9 (where the active form should predominate) and at pH 9.8 (where the inactive form should predominate). Notice, in the water solution, the characteristic fine structure in the near-UV spectrum, attributed to tryptophan and to tyrosine residues, as well as the characteristic band at ca. 260 nm (where also the disulfide chromophore contributes). Notice also, in the lower wavelength regions, the change in intensity of the 230-nm band with pH.

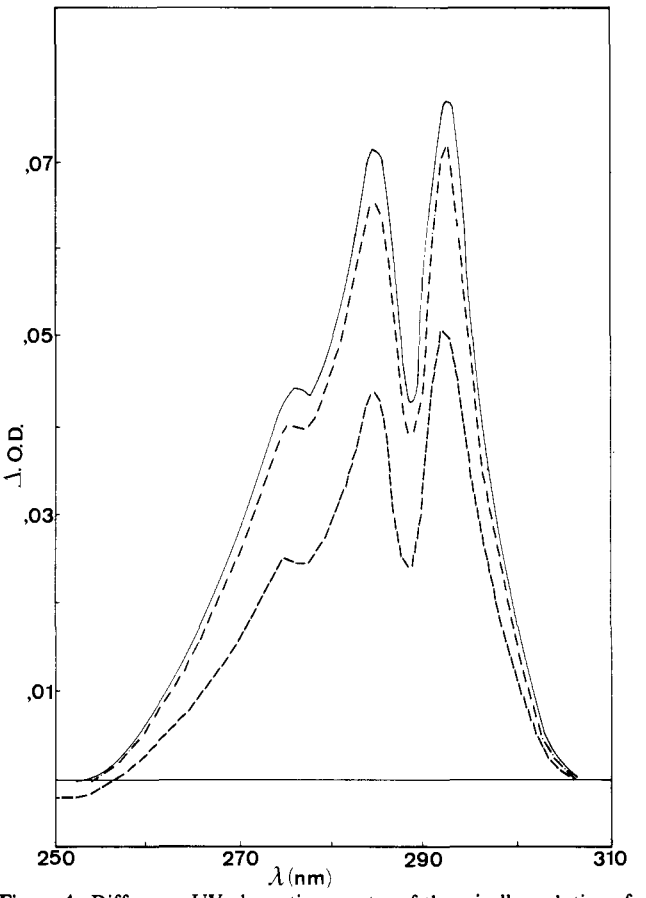

Figure 4. Difference UV absorption spectra of the micellar solution of α-chymotrypsin (11 μM overall) with respect to water, pH 7.9: —, w_0 = 6.6; - - -, w_0 = 13.5; ---, w_0 = 22.5.

Let us consider now the micellar solutions. The fine structure evidenced in the 250–320-nm region is maintained without any shift of the wavelength of the maxima, and at lower w_0 values (6.6–13.5) an increase of ellipticity is observed with respect to water solution. In the region around 260 nm, there is a drastic decrease for w_0 = 22.5. In the far-UV region, there is a significant increase in ellipticity of the 230-nm band for lower w_0 values, whereas for w_0 = 22.5 the ellipticity is less intense and it is very similar to those of the inactive α-chymotrypsin in water at high pH.

Note also that there can be marked changes in the 230-nm region without any change in the near-UV region (as exemplified by the spectrum for w_0 = 18). This confirms the interpretation that this band at 230 nm originates from the main-chain conformation[19] (helical structure) rather than from an aromatic contribution.[21]

Thus, the most interesting observation evidenced in Figure 5 is the relationship between the activity of the enzyme and the intensity of the 230-nm CD band. In particular, it looks as if the protein in the micellar environment at low w_0 has a higher helical content, which is attended by an increase in enzyme activity. In keeping with the notion that in aqueous solution chymotrypsin exists in equilibrium between two forms having different activities and different CD-properties, one could reason that in the micellar

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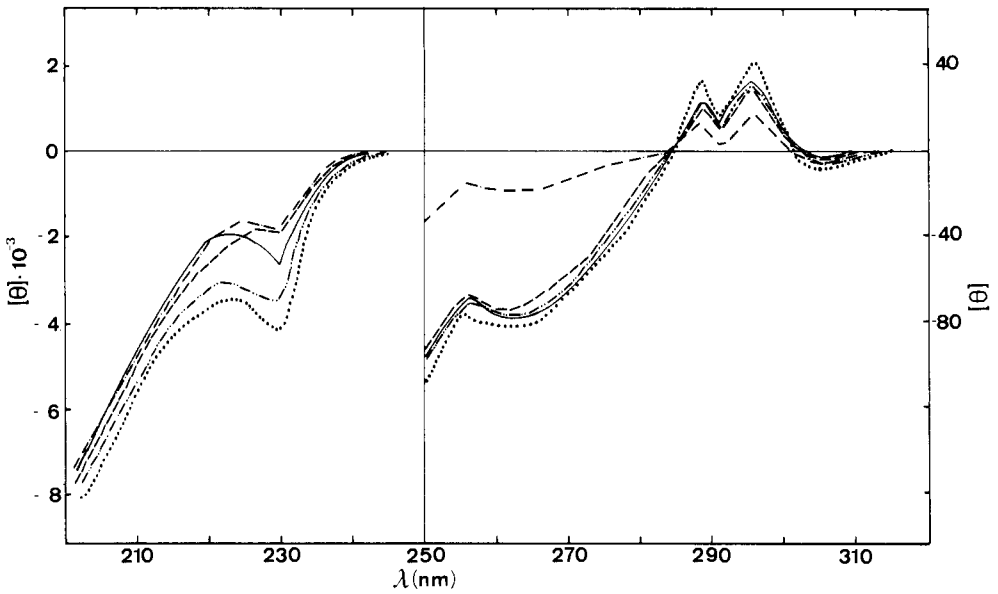

Figure 5. Circular dichroism spectra of α-chymotrypsin (overall concentration 11 μM) in water and micellar solutions at different w_0: —, water, pH 7.9; - - -, water, pH 9.8; - · - ·, w_0 = 22.5; - · · -, w_0 = 18; · · · ·, w_0 = 13.5. The CD spectrum at w_0 = 6.6 (not shown) is very similar to the spectrum at w_0 = 13.5. $|\theta|$ is the molar ellipticity per residue.

environment the conformational equilibrium is overwhelmingly shifted toward the more active form.[22]

Concluding Remarks

In this analysis of the spectroscopic properties and catalytic behavior of α-chymotrypsin in a hydrocarbon micellar solution, two observations stand out for their general relevance. One is the possibility of modulating the enzyme's activity by regulating the apparent pK of the active groups; the second one is the correlation between the "superactivity" of the enzyme micellar solution (i.e., its enhanced activity relative to an enzyme water solution) and the increased conformational rigidity.

By which mechanism does the enzyme's properties change when it is confined in the reverse micelles? At this stage of our investigations it is perhaps only safe to say that anomalous properties of the water solvent in the micelles should play a major role. The primary source of anomaly[23] is the high charge density of the surfactant layer, but it is difficult at the moment to go beyond this obvious statement and describe analytically (e.g., in terms of local dielectric constant, or water rigidity, or microviscosity) the nature of the protein's environment in the reverse micelles. Qualitatively, one could explain several of the data by assuming that water in the micelles is changed into a milieu having a lower dielectric constant and a higher microviscosity than bulk water has. This could intensify hydrogen bondings in the protein (which play a very important role for both the conformation and the activity of α-chymotrypsin[24]). In this way, Figures 2 and 5 could reflect the same phenomenon; i.e., an increase in the pK of certain amino acid residues could be correlated with an increase in the helix content, which in turn would change the conformational equilibria. The increase in the turnover number would be consistent with this picture, if one agrees that a change in the conformational equilibrium will increase the population of the more active protein species. Thus, the threefold relationship, pK changes–conformational changes–activity changes in micelles, could be satisfactorily explained and in a way which is consistent with the current ideas about this enzyme in aqueous solution (in particular, with the existence of two forms in equilibrium and with the importance of H bonds in the charge-relay system which modulates activity).

Concerning "superactivity", it should be noted that this has not been found for the other enzymes in micelles which have been investigated in detail at this time such as lysozyme or horse liver alcohol dehydrogenase. In the case of α-chymotrypsin an increase in activity has been observed not only for the amide substrate studied in this paper but also for esters.[2] This can be seen as a further support for the argument presented above that the superactivity in an intrinsic feature of α-chymotrypsin, most probably due to a peculiar conformational equilibrium. The correlation between a higher activity and a higher rigidity is also in keeping with the time–stability data reported in Figure 3. The fall off in activity of proteases with time is in fact due also to autolysis, and when this mechanism is more important, the more flexible is the macromolecule.[25] Thus, an increase in the conformational rigidity of the protein would result in a decreased probability of inter- or intramolecular autolysis. The increased stability could be also due to the fact that only one protein molecule is present inside a micelle.[26] Because of this, the intermolecular contacts among protein molecules may be limited (this may be indeed the case at low w_0 values), which would cause a limited intermolecular autolysis.

The fact that for α-chymotrypsin in reverse micelles there are conditions under which a remarkable time–stability is observed makes us expect that the same could be achieved with other enzymes. Thus, micellization would provide a new method for enzyme stabilization, which has the following two main advantages over the other methods such as enzyme immobilization: (a) catalysis of water-insoluble, lipophylic substrates is possible and (b) activity and conformation of enzymes in this new system can be investigated by traditional spectroscopic methods.

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(22) This increase of the pK of ionizable groups of the protein in reverse micelles (see Figure 2) may explain why a very good activity is obtained for the micelles also under high alkaline conditions, where in water α-chymotrypsin is inactive.

(23) The anomaly of water in the reverse micelles is apparent from proton NMR spectroscopy,[10] by the fact that water under certain micellar conditions does not freeze at subzero temperature[3,4d] and from IR data (Wells, M. A. *Biochemistry* **1974**, *13*, 4937–4944.

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(26) We thank one of the reviewers for this suggestion.

(27) **Note Added in Proof**: The group of Martinek has prepared another manuscript (*Biochim. Biophys. Acta* **1981**, *657*, 277–294) covering some other interesting aspects of enzymes in reverse micelles. We thank the authors for sending us a copy prior to publication.