Comparison of the dynamic structure of α-chymotrypsin in aqueous solution and in reversed micelles by fluorescent active-site probing

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A highly fluorescent anthraniloyl (Ant) group was covalently attached to the active site of α-chymotrypsin (CT), probably at Ser195. Ant-CT is stable at neutral pH for months, allowing a detailed fluorescence study of Ant-CT as a model protein to investigate its physical properties in 0.1 M Tris/HCl, pH 8.2, and in reversed micelles of n-octane, 0.1 M Tris/HCl, pH 8.2, and sodium bis(2-ethylhexyl)sulfosuccinate (AOT).

Steady-state fluorescence measurements of the progressive red-shift of the center of gravity of the emission band as function of degree of hydration, \( w_o \), defined as \([\text{H}_2\text{O}] / [\text{AOT}]\), indicate that the average polarity in the vicinity of the probe is approaching that of bulk water at \( w_o \geq 12 \). Time-resolved fluorescence measurements of Ant-CT in water and in reversed micelles showed that the active site has different properties in reversed micelles compared to those in water. Some specific changes at very low water content (0.6 < \( w_o < 5 \)) can be observed, which correlate with enzyme activity measurements in the same \( w_o \) region (unpublished results). These effects are, for instance, significant changes in the average fluorescence lifetime and the internal flexibility of the probe. The overall rotational-correlation time of the enzyme in AOT reversed micelles seems to be independent on \( w_o \) (5 < \( w_o < 29 \)), which suggests that the enzyme creates its own micelle.

Reversed micelles are tiny aqueous droplets, surrounded and stabilized by a monolayer of surfactant molecules, and dispersed in a water-immiscible organic solvent. Enzymes can be solubilized in reversed micelles without loss of activity. Since enzymes in reversed micelles are optically transparent, fluorescence spectroscopy has proved to be a sensitive technique to probe the dynamic structure around given fluorophores. Enzymes if a suitable fluorescent chromophore can be attached to the active site of enzymes if a suitable fluorescent chromophore can be attached in the active site. The probe must have a sufficiently long excited-state lifetime so that the flexibility of the active site, as well as the rotational mobility of the whole protein, can be determined. In addition, it must have absorption and emission spectrums well removed from those of the aromatic amino acid residues of the enzyme, especially when this protein contains many tryptophans [4]. Haugland and Stryer [5] have isolated a highly fluorescent anthraniloyl derivative of α-chymotrypsin after enzymic reaction with \( p \)-nitrophenyl anthranilate. The anthraniloyl group was covalently bound to the enzyme, probably at Ser195 of the active site (acyl enzyme). Only 1 anthraniloyl group/molecule α-chymotrypsin was introduced. The anthraniloyl derivative had no enzyme activity and was stable for months at neutral pH. The fluorescence spectral maximum is at 422 nm and the absorption maximum is at 342 nm, both well removed from those of the many aromatic residues of α-chymotrypsin. The fluorescence lifetime was measured to be 7.2 ns [5]. All these properties make this fluorescent α-chymotrypsin a very appropriate system to study protein dynamics in aqueous solution and in reversed micelles.

Rotational properties of the enzyme can be determined from time-resolved fluorescence-anisotropy experiments. There are two types of motion to be considered: fast (sub-nanosecond) internal probe motion and slower (nanosecond) overall protein rotation. If there is an increased contribution of fast internal motion upon protein incorporation into the micelles, the protein structure could be locally perturbed. A careful examination of the protein rotation in reversed micelles may reveal protein/surfactant interaction and may lead to a detailed structural characterization of the protein-containing droplet.

We have used anthraniloyl-α-chymotrypsin (Ant-CT) as a model protein to investigate its physical properties in aqueous solution and in a reversed-micellar system. We have chosen the well-studied sodium bis(2-ethylhexyl)sulfosuccinate (AOT) as surfactant for reversed micelles. The aim of this study was to answer basic questions: what is the structure and dynamic behaviour of the enzyme in the micellar system at various \( w_o \) values in comparison with these properties in bulk water? In general, the enzyme can be located either inside the water pool or bound to the surfactant interface (inserted into or peripherically bound to the interface), depending on the

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Abbreviations: Ant-CT, anthraniloyl-α-chymotrypsin; AOT, sodium bis(2-ethylhexyl)sulfosuccinate; NpAnt, \( p \)-nitrophenyl anthranilate; \( w_o \), water/surfactant molar ratio.

Enzyme. α-chymotrypsin (EC 3.4.21.1).
The work of Shapiro et al. [7] compared different models of protein solubilization in reversed micelles in case of equal size of empty water pool and enzyme globule. The latter group strongly advocated the so-called fixed-size model, in which the protein is inserted without change to the volume of the micelle. The water-shell model, on the other hand, suggests that the micelle increases in size when the protein is entrapped [6, 8]. Recent work of Rahaman and Hatton [9] using small-angle neutron scattering of AOT reversed micelles, empty and filled with z-chymotrypsin, demonstrated that the size of protein-filled micelles do not change at various degrees of hydration. Other methods that have been applied to obtain hydrodynamic radii of protein-free and protein-containing reversed micelles involved quasi-elastic light scattering [10] and fluorescence recovery after photobleaching [11]. The protein selected was myelin basic protein and the hydrodynamic radii were not very much dependent on $w_0$, but were affected by variations in the concentration of AOT at high water content.

The advantage of using sensitive time-resolved fluorescence of the active-site labelled $z$-chymotrypsin in reversed micelles is that the reversed-micellar system can be studied with very low occupancy of protein ($<1\%$). Since anisotropy data can be acquired at high dynamic range, accurate structural parameters of the protein-filled micelle can be determined. An additional advantage of using this protein derivative is that we are observing detailed events occurring in the active site of the enzyme.

**MATERIALS AND METHODS**

**Chemicals**

AOT was obtained from Sigma Chemical Co (St. Louis, USA). n-Octane was purchased from Merck (Darmstadt, FRG). The solvent was stored on a molecular sieve and filtered prior to use. Water was purified on a Millipore system. Bovine pancreatic $z$-chymotrypsin (3 x recrystallized) was obtained from Sigma. The normality of the enzyme stock solution was determined by active-site titration with $N$-trans-cinnamoylimidazole [12]. p-Nitrophenyl anthranilate (NpAnt), a generous gift of Dr A. C. M. van der Drift (Rijswijk), was synthesized according to [5, 13]. Ant-CT was prepared by the procedure of Haugland and Stryer [5]. The final solution was lyophilized and stored in small portions at $-20^\circ$C. In all experiments, the concentration of Ant-CT was 90 nM based on an absorption coefficient of 50000 M$^{-1}$ cm$^{-1}$ at 280 nm [5]. The activity of the modified enzyme was less than 5% of the activity of the native enzyme, probably due to unlabelled chymotrypsin. The assay was carried out with glutaryl-L-phenylalanine-p-nitroanilide (Merck) as substrate. All other reagents were analytical grade.

**Preparation of reversed micelles**

The micellar solutions were prepared by addition of measured volumes of n-octane and water to dry AOT. 1 µl Ant-CT solution in 0.1 M Tris/HCl, pH 8.2, was injected into 3 ml reversed-micellar organic solution. The mixture was gently shaken until a clear solution was obtained. Ant-CT solutions were prepared fresh, stored on ice and used within 4 h of preparation. The desired $w_o$ was reached by addition of a predetermined volume of 0.1 M Tris/HCl, pH 8.2. The amount of water in dry micellar solution ($w_o = 0.46$) was determined by Karl-Fischer method [14]. The highest $w_o$ was about 61 at $25^\circ$C. Above this $w_o$, the solution became turbid.

**Fluorescence measurements**

Absorption and steady-state fluorescence spectra were recorded on a Cary-14 spectrophotometer and a SLM-Aminco SPF-500C fluorimeter, respectively.

Time-resolved fluorescence and fluorescence anisotropy decays were obtained by the time-correlated single-photon counting technique [15]. Excitation was with a frequency-doubled dye laser [4-dicyanomethylene-2-methyl-6-(p-dimethylamino-styryl)-4H-pyran] giving 4-ps pulses at a repetition rate of 596 kHz. The excitation wavelength was at the maximum of the anilinoyl group in $z$-chymotrypsin at 340 nm, and the emission was monitored through a Schott KV 380 cut-off filter and a Baird Atomic interference filter having a band pass of 10 nm at 402 nm. One complete measurement consisted of measuring the polarized (parallel and perpendicular components) fluorescence decays of the reference compound (2 cycles of 20 s), those of the sample (10 cycles of 20 s), background which is sample without protein (2 cycles of 20 s) and again reference compound. We have acquired the fluorescence data using two time calibrations: 25 ps/channel and 60 ps/channel. Decay curves consisted of 1024 channels each. The pulse mimic reference compound was a pseudoazulene derivative in n-hexane [16].

All experiments were carried out at $25^\circ$C.

**Data analysis**

The total fluorescence decay, $s(t)$, and the anisotropy, $r(t)$, are fitted to distributions of exponential functions

$$s(t) = i_1(t) + 2 i_2(t) = \sum_{j} \beta_j \exp\left(-t/\tau_j\right),$$

$$r(t) = r_{01}(t) - r_{02}(t)/s(t) = \sum_{j} \beta_j \exp\left(-t/\phi_{\text{rot}}\right),$$

$s(t)$ and $r(t)$ are the quantities after deconvolution from the impulse-response function. For the distributed lifetimes ($\tau$) and correlation times, the time range was divided into 100 points equally spaced at log $\tau$ (or log $\phi$) between 20 ps and 100 ns. Analysis of $s(t)$ and $r(t)$ was performed using the commercially available maximum-entropy method (Maximum Entropy Data Consultants Ltd, Cambridge, England). A preferred solution from the feasible set is selected by maximizing the Skilling-Jaynes entropy and minimizing the $\chi^2$ statistics [17]. The advantage of this method is that a unique solution is found with no a priori knowledge of the decay model.

The fluorescence-anisotropy decay of fluorophore probes in proteins can be adequately described by the following expression which assumes two independent rotational motions: first, the rapid internal motion of the probe within the protein ($\phi_{\text{int}}$), and, second, the protein rotational motion as a whole ($\phi_{\text{rot}}$):

$$r(t) = \beta_1 \exp\left(-t/\phi_{\text{int}}\right) + \beta_2 \exp\left(-t/\phi_{\text{rot}}\right),$$

where $\beta_1$ and $\beta_2$ are the integrated amplitudes of the distributed correlation times assigned to these motions. The rapid rotational-correlation time can be calculated from the observed correlation time by applying the equation

$$\phi_{\text{obs}} = [1/\phi_{\text{int}} + 1/\phi_{\text{rot}}]^{-1}.$$
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as function of $w_o$. (----) 0.1 M Tris/HCl, pH 8.2. (C) Relative fluorescence intensity Ant-CT as function of $w_o$. (----) 0.1 M Tris/HCl, pH 8.2. (----) 0.1 M Tris/HCl, pH 8.2. (----)

is equal to unity. Depending on the degree of internal probe motion relative to protein rotation, $S$ will be less than unity. By algebraic manipulation of the pre-exponential amplitudes we can calculate the order parameter, $S$, from

$$S^2 = \beta_2 (\beta_1 + \beta_2).$$

The order parameter, $S$, depends on the angle over which the probe can move over the line of attachment to the protein (local symmetry axis) [18]:

$$S^2 = 1/2 \cos \psi (\cos \psi + 1).$$

From Eqn (6), we can estimate the value of the angle $\psi$ of displacement, responsible for the rapid internal motion. From the correlation time of restricted motion ($\phi_{int}$), one obtains the diffusion coefficient ($D_\perp$) [18]:

$$D_\perp = (1 - S^2) / (6 \phi_{int}).$$

**Structural parameters of protein-filled reversed micelles from long correlation times**

From fluorescence-anisotropy decay measurements of protein-filled reversed micelles, it is expected that the long correlation time reflects the rotational properties of the protein/micelle system in the organic solvent. Let us assume that the protein-filled micelles behave as spherical particles. According to the Stokes-Einstein relationship we have

$$\phi_{long} = \eta V_{tot} / kT = \eta [(4/3) \pi R_h^3] / kT,$$

where $\eta$ is the viscosity of the organic solvent, $k$ the Boltzmann constant, $T$ the absolute temperature, $V_{tot}$ the total volume of protein-filled micelle and $R_h$ the hydrodynamic radius,

$$R_h = R_c + 1.2 \text{ (nm)},$$

where $R_c$ is the core radius and 1.2 nm is the tail length of the AOT molecule. $V_{tot}$ is composed of

$$V_{tot} = V_{prot} + V_{shell},$$

where $V_{prot}$ is the volume of one chymotrypsin molecule ($4.16 \times 10^{-26} \text{ m}^3$) and $V_{shell}$ the volume of the remaining spherical segment of water and surfactant, including tails. Using simple geometric considerations [6], the core radius $R_c$ can be related to the total area of the droplet according to

$$R_c^2 = nA/4\pi,$$
RESULTS

Reaction of p-nitrophenyl anthranilate with \( \alpha \)-chymotrypsin in aqueous solution and in reversed micelles

The fluorescence of the anthraniloyl chromophore in NpAnt, dissolved in aqueous buffer, is completely quenched by the \( p \)-nitrophenyl moiety. The addition of ANT to \( \alpha \)-chymotrypsin leads to a large increase in the 422-nm emission peak of the anthraniloyl chromophore and to a large decrease in the 333-nm emission peak of tryptophan fluorescence of the enzyme. There is no peak or shoulder at 393 nm, which shows that the antranilic acid is not released in the course of the reaction. The existence of an isoemissive point at 379 nm confirms that only two fluorescent species in the enzyme are present. The kinetic parameters of the reaction in Tris/HCl, pH 8.2, \( k_{\text{cat}} = 0.010 \pm 0.001 \text{ s}^{-1} \) and \( K_m = 21.5 \pm 1.7 \mu M \) were calculated from the initial rate data, obtained from the visible absorption changes in liberated \( p \)-nitrophenol at 400 nm (\( e = 18000 \text{ M}^{-1} \text{cm}^{-1} \)). The range of substrate concentrations used included the \( K_m \) for the substrate. In contrast to this rapidly proceeding anthraniloylation of \( \alpha \)-chymotrypsin, the same reaction is not detected in a reversed-micellar system of 0.1 M AOT, 0.1 M Tris/HCl, pH 8.2, \( w_o = 1.45 - 11.8 \) and \([S] = 2.0 \mu M, [E] = 10 \mu M\) (substrate and enzyme concentrations are based on total volume), for periods of more than 2 h.

Steady-state fluorescence measurements of Ant-CT

The fluorescence-emission spectrum of Ant-CT was recorded in micellar solution at different \( w_o \) and compared to its spectrum in aqueous solution. An example is shown in Fig. 1A at \( w_o = 0.65 \), \( w_o = 60.1 \) and in Tris/HCl, pH 8.2. In water, the center of gravity of the emission spectrum was 417.3 nm. Upon incorporation of Ant-CT into reversed-micellar solution, a blue shift of the emission maximum was observed. With increasing water content, the center of gravity of the fluorescence spectrum of Ant-CT as a function of \( w_o \) showed a gradual red shift which at very high \( w_o \) approaches that in bulk water (Fig. 1B). The emission quantum yield decreased in parallel (Fig. 1C).

Fluorescence-lifetime measurements of Ant-CT in aqueous solution and in reversed micelles

The fluorescence decay of Ant-CT was measured as a function of hydration level (\( 0.65 < w_o < 29 \)). In Fig. 2, examples of the time-resolved experimental total fluorescence profiles are shown for Ant-CT in 0.1 M Tris/HCl, pH 8.2, and in reversed micelles at \( w_o = 0.65 \). Both fluorescence decays are heterogeneous. The fluorescence-lifetime distribution of Ant-CT in aqueous solution consists of four peaks (Fig. 3A). The main peak has a barycenter at 9.8 ns (41.4% of the total fluorescence decay). The fractional contribution of the three shorter components is 5.2 ns (29.9%), 2.2 ns (14.9%) and 0.1 ns (13.7%). In reversed-micellar solution with a very low water content (\( w_o = 0.65 \)), the image is dominated by a peak with a barycenter at 8.7 ns (99.6%). A very small contribution has the second peak (0.4%) with a barycenter at 0.1 ns (Fig. 3B). The broadening of the main peak is much higher (2.9 ns) than the corresponding peak in aqueous solution (1.0 ns). As it can be seen the peaks at 5.2 ns and 2.2 ns (Fig. 3A) are not resolved in the situation depicted in Fig. 3B. At increasing water content in reversed micelles up to \( w_o = 28.6 \), the resolution into four peaks is restored (Fig. 3C). At \( w_o = 28.6 \), the image consists of four peaks at 9.5 ns (38%), 5.8 ns (38%), 2.2 ns (17.9%) and 0.15 ns (6.7%). This distribution approaches the situation in bulk water.

The \( w_o \) dependence of the average fluorescence lifetime, \( \langle \tau \rangle \), for Ant-CT in the micellar system is presented in Fig. 4. At \( w_o = 1.5 - 2.5 \), the value of the average lifetime is constant. Upon increasing the \( w_o \) from 2.5 - 6, this value drops sharply then slowly decreases (to \( w_o = 28.6 \)), indicating that the probe is surrounded by bulk water molecules. The fluorescence intensity (Fig. 1C) showed a final reduction to 71% at high \( w_o \) compared to low \( w_o \), which is in agreement with the relative change in lifetime (Fig. 4, 73%).

Correlation-time distribution

The fluorescence-anisotropy decays of Ant-CT in aqueous solution and in reversed micelles at \( w_o = 0.65 - 29 \) were measured. Examples of the experimental polarized intensity decay curves (parallel and perpendicular) of Ant-CT in 0.1 M Tris/HCl, pH 8.2, and in reversed micelles at \( w_o = 0.65 \), are shown in Fig. 5. The two curves are initially clearly different but merge together, indicating that the anisotropy of the fluorescence vanishes because of rotational diffusion. From Fig. 5, it can be clearly observed that the curves merge together more rapidly in the case of aqueous protein solution (Fig. 5A) than for the enzyme in reversed micelles (Fig. 5B). Both polarized decay curves were globally analyzed by the maximum entropy method to obtain the distribution of correlation times. The correlation-time image of the fluorescence-anisotropy decay of Ant-CT in buffer (Fig. 6A) displays one main peak with a barycenter at 12.8 ns (97.8% of the decay) and one minor...
peak with short correlation time (0.31 ns) with a very small contribution (2.2%). In reversed micelles at the lowest \( w_o \) (0.65), both peaks are shifted to longer correlation times (Fig. 6B). The main peak is centered at 23.5 ns with 83.6% contribution and the smaller peak is located at 2.5 ns (16.4%). When the water content of reversed micelles is increased to \( w_o = 2.5 \), the short correlation peak increases to 4 ns (14.5%), followed by a decrease to 1.2 ns (12.5%) at \( w_o = 28.6 \) (Figs 6C and 7A). The longer correlation time shifts up to 45.4 ns (78.6%) at \( w_o = 9.6 \) and remains mostly independent on \( w_o \) up to 28.6 (Figs 6C and 7B).

In Fig. 8A, the displacement angles \( \psi \) (see Eqn 6) are plotted against \( w_o \). The short correlation time is longer than found in water. This indicates that the rate of reorientational diffusion \( (D_\perp) \) is smaller than in water. In Fig. 8B, the reorientational diffusion constants, as a function of \( w_o \), are presented. The smaller rate can be caused by the higher viscosity of surrounding solvent molecules, which is known to damp rapid fluctuations in proteins [21]. A pattern similar to (reciprocal) internal-correlation time (Fig. 7A) was found for diffusion coefficients \( D_\perp \) (Fig. 8B).

From the long correlation time, we are able to characterize the protein/reversed-micellar system. Eqns (8 – 11) allow us to determine the structural parameters of protein-filled droplets from the observed long correlation time (Fig. 7B). These data have been collected in Table 1. For clarity, we have included the rotation correlation times of empty droplets, experimentally determined by Vos et al. [1], into Fig. 7B, from which it can be observed that empty micelles indeed increase in size with \( w_o \) as predicted by Eqn (12).

**DISCUSSION**

Using the anthraniloyl group as fluorescent reporter group, we are able to observe only \( \alpha \)-chymotrypsin-filled micelles. The very high sensitivity of the fluorescence technique enables us to study the micellar system at very low occupancy of protein (\(<1\)%). In addition, this system is suitable to be studied at very low water content, so that changes in hydration can be monitored through fluorescence spectra and lifetimes. Also, insight into local flexibility of the active site can be obtained, as well as structural information upon incorporation of the protein molecule in reversed micelles of different water pool size from fluorescence-anisotropy data.

The physicochemical properties of the water in reversed micelles at low \( w_o \) are rather different from those of bulk
of water in the reversed-micellar system of AOT/water/heptane was investigated by both $^1$H-NMR and $^{23}$Na-NMR spectroscopy [23]. Data were interpreted by assuming highly immobilized water in small pools. In agreement with these studies our spectroscopic results demonstrate different characteristics between reversed micelles of low degree of hydration and those of higher hydration levels. The center of gravity of the fluorescence spectrum of Ant-CT in reversed micelles of very low $w_o$ shows a blue shift, in comparison to that in aqueous solution. This blue shift may be due to immobilization of the anthraniloyl chromophore in a rigid water/surfactant medium of reversed micelles of very low water content. The blue shift can also be interpreted as a retarded relaxation of water and amino acid dipoles around the fluorophore [24]. The center of gravity of the fluorescence spectrum of Ant-CT with increasing $w_o$ shows a gradual red shift approaching that in buffer, which suggests that at high $w_o$ the average polarity in the vicinity of the probe is similar to that in bulk water, indicating very rapid dipolar relaxation. Haugland and Stryer [5] found that the light-absorption maximum of Ant-CT in water is at longer wavelength than that of any model compound investigated. Methyl anthranilate has an emission maximum in water at 420 nm and in cyclohexane at 380 nm. Ant-CT has an emission maximum at 422 nm. From these spectral data, they attributed this high polarity to the presence of the carboxylate group of the aspartate residue adjacent to the reactive serine of the enzyme.

The fluorescence decay of Ant-CT in aqueous solution is complex. The total fluorescence-intensity decay of Ant-CT in water is multi-exponential. Up to four peaks can be distinguished in the lifetime distribution of Ant-CT in water, which could be attributed to the complex local environment of fluorophore and/or to the conformational heterogeneity of the protein. Each peak may represent a distinct substate of the protein with a slightly different environment of the anthraniloyl group. It can be visualized that some amino acid residue can act as quencher of anthraniloyl fluorescence (a tryptophan or tyrosine may be a good candidate). The short lifetime (0.1 ns) arises then from close proximity of the quencher to the anthraniloyl group. If they are more distant, the lifetime of this conformation would be longer. It is a well-known fact that the active site of chymotrypsin consists of distinct binding loci [25] which differ greatly in polarity. As the fluorophore is inserted in the active site of $\alpha$-chymotrypsin, a multimodal distribution of lifetimes is also to be expected since fluorescence lifetimes (and quantum yields) are somewhat sensitive to polarity. Levashov et al. [26] have carried out a comparative study of the catalytic activity of $\alpha$-chymotrypsin and the spin-label rotation frequency in the $\alpha$-chymotrypsin active center in reversed-micellar systems consisting of AOT, octane and water/organic-solvent mixtures of different viscosity. The experimental results were quantitatively interpreted in terms of a single kinetic scheme postulating the existence of the enzyme in two interconvertible forms differing in conformational flexibility, i.e. a relaxed one predominantly existing in aqueous solution, and a tense one whose proportion parallels the increase in concentration of the water-miscible organic solvent in the reverse-micellar system. Probably, the multiplex pattern of the Ant-CT lifetime distribution in aqueous solution is of similar origin owing to the coexistence of different enzyme conformers. In agreement with this concept, we observed that the lifetime distribution pattern changes after incorporation of the enzyme in reversed micelles of very low water content to an almost unimodal distribution. This indicates that the enzyme is predominantly present as a single conformer charac-

water. The apparent viscosity of water corresponds to that of a 78% aqueous glycerol solution [22]. The apparent dielectric constant in the water pool is much lower than that of bulk water, even at high water concentration. Structure and state
Fig. 6. Correlation-time distribution patterns of fluorescence-anisotropy decay of Ant-CT in different solutions. (A) 0.1 M Tris/HCl, pH 8.2; (B) 0.1 M AOT, 0.1 M Tris/HCl, pH 8.2, in n-octane at \( w_o = 0.65 \); (C) 0.1 M AOT, 0.1 M Tris/HCl, pH 8.2, in n-octane at \( w_o = 28.6 \).

The weighted residuals between calculated and observed parallel (channels 1–1000) and perpendicular (channels 1001–2000) fluorescence-intensity components are shown to demonstrate the high quality of the fit.

...terized by a long fluorescence lifetime and high fluorescence quantum yield. At increasing degree of hydration (\( w_o > 9 \)) the image of the lifetime distribution approaches the situation in buffer solution.

The rotational correlation time distribution of Ant-CT in aqueous solution provides a measure of the degree of flexibility of the active site relative to the rotation of the whole protein molecule. A unimodal, sharp peak at 12.3 ns reflects the rotational-correlation time of protein in buffer solution which is expected for a protein of this size. The internal probe motion is presented by a peak at 0.31 ns, but the contribution is extremely small. From the relative contributions of the two types of motion one can derive the so-called order parameter \( S \) of the probe bound to the protein, and from this one can obtain the angular displacement corresponding with the fast motion (see Materials and Methods). From the analysis of the correlation-time distribution, it was found that \( S = 0.988 \) and \( \phi = 10.1^\circ \). It can then be concluded that the anthraniloyl fluorophore has very small free volume to move inside the active site and that it is rigidly attached to the protein and rotates together with the whole protein molecule. The value of rotational correlation time for an anhydrous, rigid sphere of the size of \( \alpha \)-chymotrypsin molecule is predicted to be 10 ns [27]. The difference between predicted and experimental correlation times arises because chymotrypsin is either not a sphere or it is hydrated to a large extent.

In a micellar system of very low hydration level, both short and long rotational correlation times for Ant-CT change considerably. The contribution of the shorter correlation time in the distribution increases, indicating that the active site created some free volume in which the probe can reorient. For instance, at \( w_o = 0.65 \), we found an order parameter \( S = 0.914 \) leading to a displacement angle of 27.5°. Such an effect may originate from subtle changes in active site conformation, e.g. the rupture of an H bond. The increase in short correlation time observed in the range \( 0.65 < w_o < 5 \) can be interpreted as a retardation of the segmental flexibility of the anthraniloyl group. These specific changes at very low water content correlate with enzyme activity measurements (unpublished results).

Rahamam and Hatton [9] have determined the micellar radii of empty and chymotrypsin-filled reversed micelles using small-angle neutron scattering. Let us consider two extreme cases to compare results obtained with two completely different techniques. Rahamam and Hatton [9] found for \( w_o = 5 \) a micellar-core radius of 2.0 nm corresponding with a long correlation time of 33 ns, according to Eqns (8) and (9); for
Fig. 7. The $w_0$ dependence of barycenters obtained from the short (A) and long (B) correlation-time distribution of Ant-CT in AOT reversed micelles. Duplicate measurements of the internal correlation time (A) are given for lower $w_0$ values ($0.65 < w_0 < 5$). (---) 0.1 M Tris/HCl, pH 8.2 (B). Also, correlation times for empty micelles (taken from [1]) are given (○).

For $w_0 > 15$, this radius was 3.4 nm, leading to a correlation time of 47 ns. These values are in excellent agreement with the data presented in Fig. 7B. We therefore sustain the conclusion reached in [9], that there is no variation of protein-filled micellar size except at low hydration level. This experimental finding is in contrast to the model of Caselli et al. [8] who predicted an almost linear dependence of the core radius of protein-filled micelles with $w_0 = 5 - 25$.

The fact that the enzyme creates its own micelle may have implications for the catalytic behaviour. In first approximation one can expect, that the enzyme activity will be unaffected at various water contents, because the size of the enzyme-filled droplet is the same between $10 < w_0 < 30$. However, it should be noticed that the shape of activity/$w_0$ profiles

Table 1. Structural parameters of Ant-CT-filled AOT reversed micelles from the long rotational-correlation time.

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<th>$R_{\text{fit}}$</th>
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<td>4.7</td>
<td>3.5</td>
<td>366</td>
<td>3.6</td>
</tr>
</tbody>
</table>

$R_{\text{fit}}$, Eqn (8); $R_{\text{fill}}$, Eqn (9); $n$, Eqn (11); $V_{\text{shell}}$, Eqns (8) and (10).
is not only a characteristic property of the enzyme alone, but also depends on the nature of the substrate [28] and on the overall enzyme concentration [29]. In addition, although variation of the water content of the system does not affect the size of enzyme-filled reverse micelles, it will affect the size of substrate-filled reverse micelles and hence the exchange rate between micelles [30]. It has been shown that the differences in intermicellar-exchange rates might influence the observed kinetic constants [31]. Furthermore, in many cases bell-shaped curves can be explained by the use of non-saturating substrate concentrations [32].

Finally, we have presented novel information on proteins in systems of very low water activity, i.e. in reverse micelles with 0.65 < w_a < 5. The enzyme can be incorporated in AOT reverse micelles with the minimum amount of water and forms a compact rotating particle, surrounded by some 170 surfactant molecules (Table 1), characterized by a correlation time of 23 ns. A slight increase in water content makes the protein/micelle system swell. As suggested in Table 1, the number of surfactant molecules increases upon hydration. It can be envisioned, however, that the surfactant head groups become hydrated first, and this is accompanied by an increase in area without changing the number of surfactant molecules surrounding the protein. The swelling process is essentially complete at w_a = 2.5—5. After hydration of the surfactant head groups, protein hydration sets in which seems to be complete at w_a = 10, i.e. within a relatively small range of w_a indicating cooperative binding of water molecules to the protein. It should be noted that full enzyme activity is developed within the same w_a range strongly suggesting that proper hydration of the enzyme is the determinant for catalytic activity [33].

From the different fluorescent parameter values as function of w_a, it can be concluded that enzyme-containing reverse micelles change in size, which is accompanied by a considerable change in active-site conformation.

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