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Activity and conformational changes of α-chymotrypsin in reverse micelles studied by spin labeling

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α-Chymotrypsin (CT), spin-labeled at the active site by using an acylating label which constitutes a substrate for this protein, has been investigated in reverse micelles formed by AOT in isooctane. The electron spin resonance spectra provided information on conformation, dynamics and deacylation activity. The dynamics of the label bound to CT appears to be more hindered in reverse micelles than in aqueous solution, probably owing to the effect of the micellar environment on protein conformation. The deacylation rate in reverse micelles does not show the characteristic bell-shaped dependence on water content which is generally found for CT enzymatic activity.

α-Chymotrypsin; AOT; ESR; Reserve micelle; Spin labeling

1. INTRODUCTION

α-Chymotrypsin (CT) has been widely studied in reverse micelles with particular attention to its enzymatic activity and to conformational changes [1–5]. In our group, proteins in reverse micelles have been studied by ESR spin labeling [6]. Spin labeling constitutes a rewarding tool in investigating the dynamics, conformation and micro-environment of proteins [7] and it has proven to be useful in characterizing proteins hosted in reverse micelles [5,6].

In the present communication we report ESR results obtained on spin-labeled CT in reverse micelles constituted by AOT in isooctane. CT was labeled at the active site by using the acylating spin label **I** (3-carboxy 2,2,5,5-tetramethyl pyrrolidine-*N*-oxyl-*p*-nitrophenyl ester) which constitutes a substrate for this protein [8–10]. By using this label it was possible to obtain information on the dynamics and conformation and to measure the deacylation rate of CT in reverse micelles. The results obtained show that: (1) the rotational correlation time of the label bound to CT is significantly higher than in aqueous solution owing probably to the influence of the micellar matrix on CT conformation; (2) the deacylation rate in reverse micelles does not show the characteristic bell-shaped dependence on water content which is generally found for CT enzymatic activity [2]. It is, in fact, approximately constant and about 1.2 times the value in buffer, at $w_0=([H_2O]/[AOT]) \gtrsim 7$, while is smaller than in buffer at $w_0 \lesssim 7$. This result is discussed in connection with the possible role of the rate of micellar diffusion in the organic solvent on protein enzymatic activity [11].

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2. MATERIALS AND METHODS

2.1. *Chemicals*

CT (from bovine pancreas, 3× crystallized and lyophilized) and **I** (3-carboxy 2,2,5,5-tetramethyl pyrrolidine-*N*-oxyl-*p*-nitrophenyl ester) were purchased from Sigma. AOT was a kind gift of Dr M. Waks (Paris) and was used, after accurate drying, without further purification. Isooctane, spectroscopic grade, was purchased from Carlo Erba (Italy).

2.2. *Labeling of CT and measurement of the deacylation rate*

The CT-catalyzed hydrolysis of a number of esters involves the following steps [8,10]:

$$E + S \rightleftharpoons (ES) \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

where E is the enzyme and S the substrate. In the first step of the reaction the ester binds to the enzyme giving rise to the complex ES (not stable) which decays with a rate constant k_2 producing the acyl enzyme (ES') and the alcohol P_1. ES' decays with rate constant k_3 to give the acid (P_2) and the free enzyme. In aqueous solution, the deacylation is the rate-limiting step of the reaction, since $k_2 \gg k_3$. This reactive path is valid when the substrate (S) is constituted by the spin label **I**: at acidic pH, k_3 is small and the acyl enzyme (ES') can be isolated [8]. The complex ES' shows an ESR spectrum which is characteristic of a strongly immobilized label. The final reaction product P_2 shows, instead, a spectrum of freely rotating nitroxide. The time dependence of this last signal is used to measure k_3 [8].

According to [9], the labeling of CT was carried out in acetate buffer, 100 mM, pH 4.7. The unreacted label was removed by ultrafiltration through a C20K spectrum membrane; the purification was carried out by washing with glycine-HCl buffer, 30 mM, pH 3.2. This value of pH was sufficiently acidic to allow isolation of the acyl

enzymes (ES'). The stock protein solution, after purification, was at pH 3.2. In order to measure the deacylation rate in aqueous solution at pH 7.9, aliquots of the stock protein solution were diluted three times with buffer at pH 7.9 (Tris, 70 mM).

2.3. *Preparation of reverse micellar solutions*

Dry reverse micellar solutions were prepared by dissolving the required amount of AOT in isooctane to obtain a final concentration of 100 mM. In order to obtain the CT containing micellar solution at pH 7.9 with a given value of w_0, we proceeded adding buffer at pH 7.9, accounting for 2/3 of the required volume of water, and stock protein solution accounting for the remaining 1/3. After gentle and quick shaking, the micellar solution was transferred to a 0.5 mm quartz tube for ESR measurements. For deacylation rate measurements, the protein concentration in reverse micelles was kept in the range 4–10 μM. All experiments were repeated.

2.4. *Spectroscopic measurements*

ESR spectra were recorded at 9.2 GHz and 25°C on a Varian E-112 spectrometer equipped with a Varian variable temperature controller. The spectrometer was also equipped with a home-made data acquisition and control system which allows a significative reduction of experimental time with respect to the standard analogical spectral recording [12,13]. For the measurements of deacylation rate, only a small portion of the ESR spectrum (corresponding to the line W_1 in Fig. 1) was acquired at a repetition rate of (1/30) s^{-1} for 15–20 minutes (corresponding to about 5 times the exponential time constants).

2.5. *Data Analysis*

Spectra were simulated by using the Freed algorithm [14]. The elements of the nitroxide hyperfine (A) and electron Zeeman (g) tensors, which are required for computing rotational correlation times, were obtained by simulating the spectrum of the complex ES' at −60°C in 50% (v/v) glycerol buffer. The deacylation rate constant, k_3, was calculated by using a three parameter least-squares fitting procedure to a single exponential decay of the experimental data.

3. RESULTS AND DISCUSSION

3.1. *Dynamics and conformation*

Fig. 1 shows the ESR spectrum of CT in buffer at pH 3.2. The spectrum contains a large contribution from a strongly immobilized label (arrows S), due to the acyl enzyme (ES'), and a small contribution from a weakly immobilized spin label (arrows W), due to a small percentage of the enzymatic reaction product P_2 [8]. Spectra recorded at pH 7.9 did not show significant differences in the distance between the outer hyperfine extrema ($2A_{zz}$, which is sensitive to the label dynamics) with respect to those recorded at pH 3.2. From the simulation of the strongly immobilized component, the rotational correlation time (τ) of the complex ES' is found to be 9.2 ns, in agreement with the value calculated for the tumbling of a 25 kDa, spherical protein with 0.3 g of hydration water *per* g of protein. If we assume that CT has a spherical shape, this implies that the label is rigidly bound to the protein [9].

Fig. 2 shows some representative spectra of acyl CT in reverse micelles. The spectra in reverse micelles show,

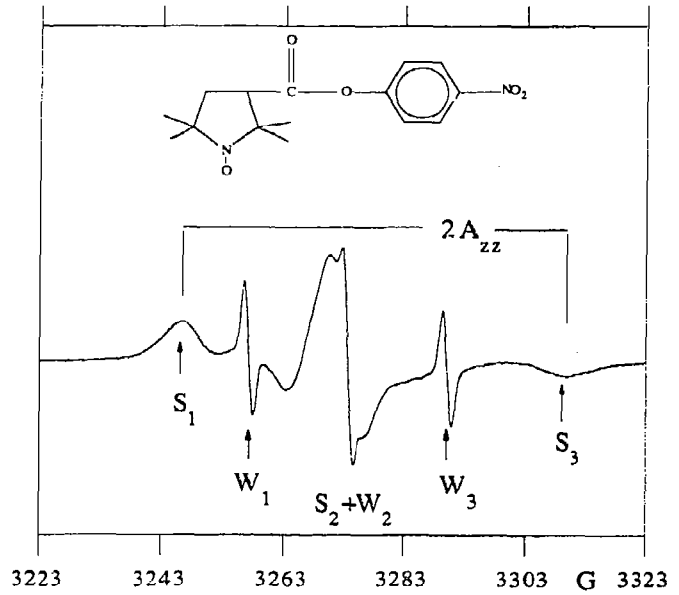

Fig. 1. ESR spectrum of acyl CT recorded in buffer at pH 3.2, 25°C and 5 mW microwave power. The formula of the spin label I is also shown.

with respect to the spectrum in buffer, different values of $2A_{zz}$ and a greater contribution coming out from the reaction product P_2. The spectra reported in Fig. 2 were obtained by using a buffer at pH 3.2[a]. Spectra of acyl CT in reverse micelles recorded at pH 7.9 did not show significant changes in the values of the parameter $2A_{zz}$ with respect to pH 3.2. Table I shows the values of the parameter $2A_{zz}$ and the values of τ, calculated by simulating the strongly immobilized component of the experimental spectra, as a function of w_0. The rotational correlation times measured in reverse micelles are higher than in water. In addition it must be pointed out

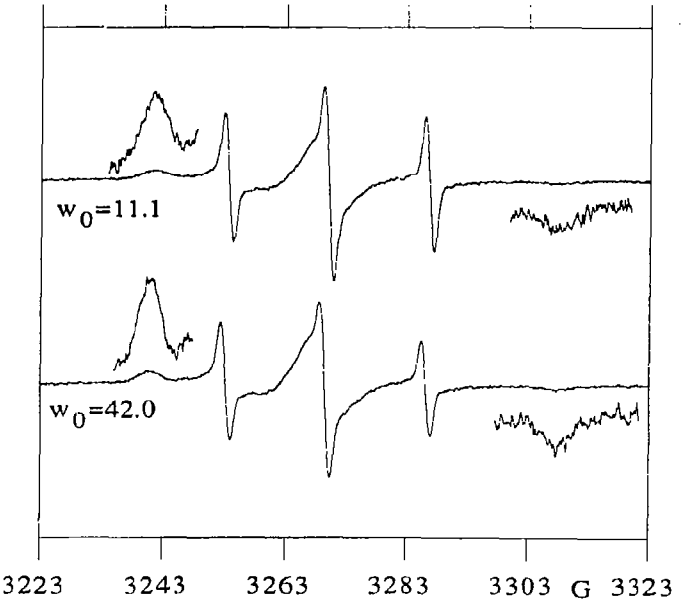

Fig. 2. ESR spectra of acyl CT in reverse micelles, recorded at pH[a]=3.2, 25°C and 5 mW microwave power.

[a]In this paper pH is the pH of the stock aqueous solution injected into the AOT-isooctane system. The local pH in the micellar water pool can be different from the pure buffer value [2].

Table I

$2A_{zz}$ and τ for the complex ES′ in reverse micelles and in buffer

w_0	$2A_{zz}$/G	τ/ns
5.6	67.5	20.8
11.1	67.8	22.6
16.7	67.5	20.8
42.0	67.2	22.0
Buffer	63.4	9.2

that these values may be an underestimate of the rotational correlation time of the label bound to CT. In fact the decreased polarity probably experienced by the label in reverse micelles may lead to a decrease in the nitroxide hyperfine tensor (A) components. When the spectra are calculated by using the components of A determined in buffer (as we did in the present case) an underestimate of τ can be introduced [6]. We can therefore conclude that the rotational correlation time of the acyl enzyme is significantly higher than in buffer even at high w_0, where the physical properties of the micellar water are similar to those of bulk water and the rotation of the whole micelle in the organic solvent does not contribute. It has been suggested that CT exists in aqueous solution in two equilibrium forms, the highly reactive, tense form (T) and the relaxed form (R) with low activity [4]. In aqueous solution the presence of a specific substrate shifts the equilibrium to the T form [4]. On the basis of activity measurements with a non-specific substrate in reverse micelles solvated by a mixture of water and water miscible organic solvents [4] and spin labeling experiments [5] it has been hypothesized that the micellar matrix may fix the highly reactive, conformationally stressed form of CT, even in the absence of a specific substrate. The finding that the rotational correlation time of the label bound to CT is higher in reverse micelles than in aqueous solution could mean that the micellar matrix influences the protein conformation also after the binding of a specific substrate. CD studies have shown an increased content of α-helix in reverse micelles with respect to aqueous solution [2]. Interactions between the protein and the micelle can, in addition, hinder its rotation. All the above mentioned effects could contribute to a decreased mobility of the label with respect to the protein structure, as well as of the protein itself, in reverse micelles compared to water.

3.2. *Deacylation rate*

The deacylation rate (k_3) of CT in aqueous solution was measured by monitoring the intensity of line W_1 of the acyl enzyme spectrum (see Fig. 1) after an abrupt change of pH to 7.9. The intensity of W_1 showed a single exponential time dependence with rate constant $k_3=3.6\pm0.1\ 10^{-3}\ s^{-1}$. The time dependence of line W_1 was

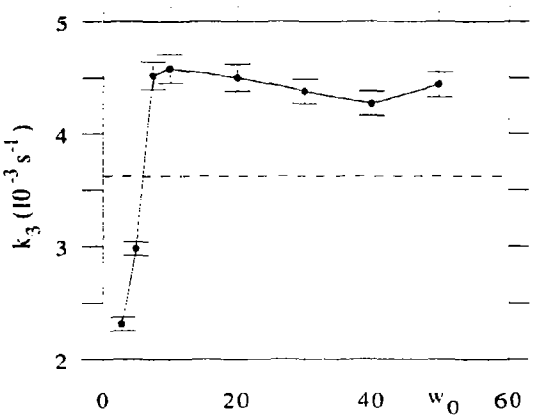

Fig. 3. Deacylation rate constant in reverse micelles as a function of w_0 at pH* 7.9. The dotted line represents the value of the rate constant in aqueous solution. The continuous line is only a visual guide.

used to measure the deacylation rate also in reverse micelles; it showed a single exponential behaviour at each investigated value of w_0. Fig. 3 shows the dependence of k_3 on water content. At high w_0 ($w_0 >\approx 7$) the reaction rate k_3 is approximately constant, within the experimental error, and assumes a value which is about 1.2 times the value in buffer. At low w_0 ($w_0 <\approx 7$) k_3 is strongly dependent on water content and increases with increasing w_0.

The present result is different from most of the previously reported data on CT activity in reverse micelles, which generally show a bell-shaped dependence on the value of w_0 [2,15]. In reverse micellar solutions, the first step of an enzymatic reaction, i.e. the formation of the complex enzyme-substrate, can occur only after two micelles, one containing the enzyme and the other one containing the substrate, have collided [11]. The collision probability decreases with increasing w_0 since it depends on the diffusion rate of the micelles in the organic solvent (i.e. on the micellar size). At high w_0, the decrease in the diffusion rate of the whole micelle may justify the decrease of the enzymatic activity and its bell-shaped profile ([11] and S. Ferreira and E. Gratton personal communication). In the present experimental conditions the enzyme with its bound substrate (the acyl enzyme) is directly inserted in reverse micelles. The first step of the enzymatic reaction, i.e. the binding between the enzyme and the substrate, therefore, does not occur. This could explain the observed trend of k_3: the acyl enzyme is able to deacylate itself as soon as it is surrounded by a sufficient amount of water and afterwards its deacylation activity remains, practically, constant. In this particular case the presence of water is necessary because it constitutes a substrate in the deacylation reaction. The value of w_0 at which the CT deacylation rate reaches the plateau value ($w_0 \approx 7$) could seem surprisingly small in the light of ref. 16. It has been in fact shown that in water containing AOT-Isooctane reverse micelles, 13 water molecules per 1 AOT molecule

are associated (more or less strongly) with AOT or Na^+ [16]. However, in the presence of proteins, a redistribution of water between unfilled and filled micelles generally occurs [17]. As a consequence of this redistribution, the protein containing micelles have a water content significantly larger than the original. In particular, an increase in the 'true' w_0 was found for CT containing reverse micelles constituted by AOT in Isooctane.

Although the rate constant k_3 represents only one step of the enzymatic reaction, we believe that the present finding can have a general relevance for understanding enzymatic activity in reverse micelles.

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