Superactivity and conformational changes on α-chymotrypsin upon interfacial binding to cationic micelles

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The catalytic behaviour of α-CT (α-chymotrypsin) is affected by cationic micelles of CTABr (hexadecyltrimethylammonium bromide). The enzyme–micelle interaction leads to an increase in both the V_{max} and the affinity for the substrate *p*-nitrophenyl acetate, indicating higher catalytic efficiency for bound α-CT. The bell-shaped profile of α-CT activity with increasing CTABr concentrations suggests that the micelle-bound enzyme reacts with the free substrate. Although more active with CTABr micelles, the enzyme stability is essentially the same as observed in buffer only. Enzyme activation is accompanied by changes in α-CT conformation. Changes in tertiary structure were observed by the increase in intensity and the red shift in the α-CT tryptophan fluorescence spectrum, suggesting the annulment of internal quenching and a more polar location of tryptophan residues. Near-UV CD also indicated the transfer of aromatic residues to a more flexible environment. CTABr micelles also induces an increase in α-helix, as seen by far-UV CD and FTIR (Fourier-transform infrared) spectroscopies. The far-UV CD spectrum of α-CT shows an increase in the intensity of the positive band at 198 nm and in the negative band at 222 nm, indicating an increased α-helical content. This is in agreement with FTIR studies, where an increase in the band at 1655 cm^{-1}, corresponding to the α-helix, was shown by fitting analysis and difference spectroscopy. Spectral deconvolution indicated a reduction in the β-sheet content in micelle-bound α-CT. Our data suggest that the higher catalytic efficiency of micelle-bound α-CT results from significant conformational changes.

Key words: chymotrypsin, circular dichroism (CD), enzyme activation, fluorescence, Fourier-transform infrared (FTIR), micellar enzymology.

INTRODUCTION

Different properties of cytosolic enzymes, such as rates and mechanism of reactions, are usually determined in solutions containing very low concentrations of protein, substrate and salts. However, in the living cell, where biochemical reactions take place, a very different condition is found where a significant fraction of its volume is 'crowded', since it is occupied by several different solutes, macromolecules and membranes [1,2]. Therefore, an accurate evaluation of the physiological role of a particular reaction characterized *in vitro* is done by considering the possible influence of crowding and/or confinement upon the reaction [1].

Studies on the effects of microheterogeneous media and organic solvents on catalytic properties of cytosolic enzymes can be helpful to the understanding of the mechanisms of catalysis in the living cell. The introduction of the micellar enzymology [3,4] gave a new perspective to the study of kinetic properties of cytosolic enzymes in a system that resembles the *in vivo* condition better than the commonly used aqueous diluted solution.

Most of the research in micellar enzymology has been done by encapsulation of enzymes into reversed micelles [3–5]. They can be defined as optically clear water-in-oil microemulsions in a ternary system containing surfactant, small amounts of water and organic solvent, forming thermodynamically stable spherical water droplets [5]. Their size increases with the water content which is expressed by the hydration ratio (W_0), the molar ratio between water and surfactant in the reversed micelle solution. Since they present an inner cavity, it is possible for proteins to be entrapped in a polar core. The activity of many soluble enzymes increases when solubilized in reversed micelles [3,4,6–9], where the superactivity usually has a bell-shaped dependence on W_0. In some cases, the maximum activity is observed when the reversed micelle is slightly bigger than the enzyme [7].

Aqueous micelles can also be used as a biomimetic system. They are dynamic systems spontaneously formed by the aggregation of a surfactant solubilized in water [10,11]. The formation of aqueous micelles occurs at a particular concentration of surfactant known as the cmc (critical micelle concentration). Above the cmc, both micelles and monomers are present, and the addition of more surfactant molecules will result in an increase in the number of micelles while the concentration of monomeric surfactant remains essentially the same [10,11]. For ionic micelles, a large fraction of counterions is located at the interfacial region between the non-polar core and the aqueous phase, a significantly hydrated region called the Stern layer [11].

The effect of aqueous solutions of surfactants on organic reaction kinetics has been extensively studied for many years [12–14]. Although this field is known as 'micellar catalysis', aqueous micelles can act as catalysts or inhibitors, depending on the nature of the reactants and surfactants (cationic, anionic or neutral) forming the micelles [14]. The fact that reaction rates exhibit a maximum at a certain surfactant concentration can be explained by theoretical models developed for the treatment of micellar catalysis [12,13]. These models consider both the partitioning of the substrate and the ion exchange at the interface since the formation of micelles causes an increase in the rate due to the local concentration effect of reactants [12,13].

Abbreviations used: cmc, critical micelle concentration; α-CT, α-chymotrypsin; CTABr, hexadecyltrimethylammonium bromide; FTIR, Fourier-transform infrared; PNPA, *p*-nitrophenyl acetate.

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Based on similar concepts, Viparelli et al. [15] described theoretical models for enzyme superactivity in aqueous solutions of surfactants considering a system of three pseudophases: free water, bound water and micelle core. According to these models, only when enzyme–micelle interactions are included in the formulation does the reaction rate exhibit a bell-shaped dependence on surfactant concentration. For such cases, the theoretical model assumes that the enzyme conformation as well as its catalytic properties are affected by interactions with the micellized surfactant. In the absence of an enzyme–micelle interaction the model predicts a tendency for a monotonic increase or decrease in the calculated reaction rate as a function of surfactant concentration [15]. Fitting of the experimental data to these models allows an appropriate analysis of enzyme superactivation, since it indicates if the bound enzyme preferentially reacts with bound or free substrate [15].

The activity and stability of soluble enzymes in aqueous micelles is closely related to the chemical nature of the surfactant [16–20]. Therefore, the observed effects depend on the type of interaction, which can be modulated by changes in pH or ionic strength. These studies with different enzymes showed a correlation between the changes in the kinetic and some conformational property of these proteins as studied by fluorescence [17–19], UV-difference [17] or ESR [16] spectroscopy.

Here we show that an increase in α-CT (α-chymotrypsin) activity by aqueous micelles of the cationic surfactant CTABr (hexadecyltrimethylammonium bromide) is related to changes in both the secondary and tertiary structures of the enzyme. α-CT is a 25 kDa soluble protease which is a well-known model enzyme studied with reversed [6,8,9] and aqueous [18,20] micelles, as well as in non-aqueous enzymic media [21,22]. The studies with reversed micelles showed that both the activity [6] and the stability [8,9] have a bell-shaped profile as a function of the hydration ratio (W_0). Although α-CT is less stable when incorporated into reversed micelles [8], the application of high hydrostatic pressure or glycerol stabilizes the enzyme against inactivation [9].

Our results indicate that the α-CT interacts with the surfactant only in the aggregated state, since no effect on the kinetic or conformational properties of α-CT was observed with monomeric CTABr. This is an indication that interfacial binding is a requirement for the enzyme activation.

EXPERIMENTAL

Assay for α-CT activity and determination of kinetic parameters

The catalytic activity of 10 μM α-CT (E.C. 3.4.21.1; type II from bovine pancreas, Sigma) was determined at 25 °C by the rate of PNPA (*p*-nitrophenyl acetate; Sigma) hydrolysis in 20 mM Tris/HCl, pH 7.5, in the absence and in the presence of 0.05–60.0 mM CTABr (Merck Indústrias Químicas, Rio de Janeiro, Brazil). The PNPA concentration in the assays was usually 2 mM. Rates of reactions were determined by following the formation of *p*-nitrophenolate by absorbance at 410 nm. Data collection and analysis were done in a Hitachi U-2000 double-beam spectrophotometer equipped with a signal processing and control system for the direct calculation of reaction rates. Similar values for the absorption coefficient (ε) of the *p*-nitrophenolate were found in the absence and presence of CTABr ($\varepsilon_{410} = 13\,400 \pm 460\ M^{-1} \cdot cm^{-1}$). Non-catalysed hydrolysis of PNPA was negligible at this temperature, with or without CTABr. The K_m for PNPA was determined at room temperature by measuring the steady-state activity of the enzyme with PNPA ranging from 0.05 to 0.5 mM in the absence and presence of 0.2, 2.0 or 20.0 mM CTABr. k_{cat} was calculated from the rate of reaction, taking into account the enzyme concentration in the assays. CTABr was used after recrystallization in methanol/acetone (1:3, v/v) according to Oliveira et al. [23].

Substrate binding to micelles

The partition of the substrate PNPA was determined according to Sepulveda et al. [24], assuming a thermodynamic equilibrium for the substrate distribution between the bulk water and the micellar aggregate. The association constant, K_S, is defined as:

$$S_F + D_N \underset{K_S}{\rightleftharpoons} S_M \quad (1)$$

where S_F and S_M represent the free and micelle-bound substrate (PNPA), respectively, and D_N is the concentration of micellar surfactant, determined from the difference of the total concentration of CTABr from the cmc. The cmc of CTABr was determined at 25 °C by isothermal titration calorimetry by using a MCS-ITC from MicroCal according to Paula et al. [25]. In the conditions used here (20 mM Tris/HCl, pH 7.5) the cmc for CTABr was 0.53 ± 0.03 mM.

K_S was calculated using eqn 2:

$$A_{280} = (\varepsilon_w + \varepsilon_M K_S[D_N])/(1 + K_S[D_N]) \quad (2)$$

where A_{280} is the absorbance at 280 nm, and ε_w and ε_M are the absorption coefficients of PNPA in buffer and in the presence of 20 mM CTABr, respectively. The association constant for PNPA binding to CTABr micelles ($K_S^{CTABr-PNPA}$) was 56 M^{-1}.

α-CT stability

α-CT (40 μM) was incubated at 25 °C in 20 mM Tris/HCl, pH 7.5, in the absence or in the presence of CTABr (0.2, 2.0, 10.0, 30.0 and 60.0 mM). After equilibration of the incubation medium at 25 °C, the enzyme was added and, at regular time intervals, samples (500 μl) were withdrawn in order to measure the enzyme activity as described above by the addition of PNPA. The residual α-CT activity was calculated as a percentage of the original activity (considered as 100 %), obtained at $t = 0$ min incubation.

Steady-state fluorescence

Tryptophan intrinsic fluorescence spectra were obtained from 450 to 300 nm at 25 °C in a Hitachi F-3010 spectrofluorimeter equipped with a temperature-control device. The excitation wavelength was 295 nm, with bandwidths of 5 nm for excitation and emission. The spectra of 5 or 10 μM α-CT were obtained at different incubation times of the protein in 20 mM Tris/HCl, pH 7.5, in the absence or presence of increasing CTABr concentrations in the monomeric (0.1 and 0.2 mM) or micellar (2.0, 4.0, 20.0 and 30.0 mM) states. Slow changes in the fluorescence spectrum of α-CT were observed up to 20 min incubation with CTABr micelles. After that, no significant changes were observed in the fluorescence spectra recorded up to 2 h incubation with the micelles. Therefore, all spectra were recorded after 30 min incubation to allow sample equilibration. Spectra of buffer alone and CTABr in buffer were subtracted from that obtained with α-CT to avoid scattering effects.

CD

Far-UV CD spectra of 10 μM α-CT were obtained from 185 to 260 nm in a JASCO J-701 (Jasco Instruments, Tokyo, Japan) spectropolarimeter with a cylindrical 0.1 cm quartz cell at the same conditions used for fluorescence. The spectra were recorded

without accumulation, and smoothed with an FFT (fast Fourier-transform) filter, after 30 min incubation with CTABr micelles to allow sample equilibration as stated above. The concentrations of CTABr were 0.2, 2.0 or 20.0 mM. For near-UV CD measurements, the spectra of 300 μM α-CT in a cylindrical 1 cm quartz cuvette were recorded from 250 to 320 nm as an average of eight scans, and smoothed with an FFT filter. Far-UV and near-UV CD spectra of surfactant solutions were subtracted to eliminate background effects due to monomers or micelles. The scan rate was 50 nm · min^{-1}, with bandwidths of 0.2 nm in all measurements. Quantitative prediction of the secondary structure was done by deconvolution of the far-UV CD spectra using the Selcon program [26]. The RMS (root mean square) was less than 1 % for all deconvolutions. The far-UV CD spectra of α-CT obtained with 20 mM CTABr were not deconvoluted, due to a high noise/signal ratio.

FTIR (Fourier-transform infrared) spectroscopy

FTIR spectra were obtained in a Nicolet Nexus instrument equipped with a deuterated triglycine sulphate KBr detector. The sample was placed in a cell for liquid samples with CaF_2 windows and 100 μm Teflon spacer. The instrument was purged with dry N_2 to decrease atmospheric water vapour. When necessary, residual water vapour was subtracted prior to deconvolution or spectral subtraction. We obtained 100 interferograms/spectrum at room temperature with a nominal resolution of 2 cm^{-1}. Typically, 0.2 mg of α-CT was dissolved in 30 μl of ^2H_2O buffer (20 mM Tris, p^{2}H 7) for 24 h to allow deuterium exchange of the amide protons. Deuterated α-CT was incubated with 3 or 30 mM CTABr for 2 h to allow sample equilibration. Similar results were obtained in both cases.

Fourier-transform self-deconvolution of the unsmoothed spectra was performed with Omnic 5.0 software using a triangular apodization function, a bandwidth HWHH (half-width at half-height) = 18 cm^{-1} and line-narrowing factor K = 2. Identification of overlapping bands was carried out with second-derivative analysis of deconvolved spectra. The components in the amide I′ region of the original (K = 1) spectrum were fitted with Gaussian bands. The bandshape was fitted by varying the peak height, the bandwidth or the peak position, one at a time in this order. Finally, the parameters were optimized by varying both the band intensities and bandwidths. Difference spectra were obtained from normalized non-deconvolved spectra (integrated area, 1 cm^{-1}).

RESULTS AND DISCUSSION

α-CT activation

The activity of α-CT was measured in the presence of CTABr micelles by determining the rate of enzyme-catalysed hydrolysis of PNPA at 25 °C. The cmc for CTABr under the conditions used here was determined by isothermal titration calorimetry (cmc = 0.53 ± 0.03 mM). Although the surfactant in the aggregate state is the major form above the cmc, monomeric surfactant is also present in the aqueous micellar system [10]. Therefore, the α-CT activity was measured in two concentration ranges of CTABr: from 0.01 to 0.5 mM where only monomeric surfactant is found, and from 2.0 to 60 mM where micelles are present. It is important to note that CTABr did not induce non-catalysed hydrolysis of PNPA in the absence of enzyme.

Figure 1(a) shows that while monomeric CTABr did not affect α-CT activity, there was up to an 80 % increase in the rate of PNPA hydrolysis in the presence of micelles as compared with the rate obtained in buffer only. α-CT activation presented a bell-shaped

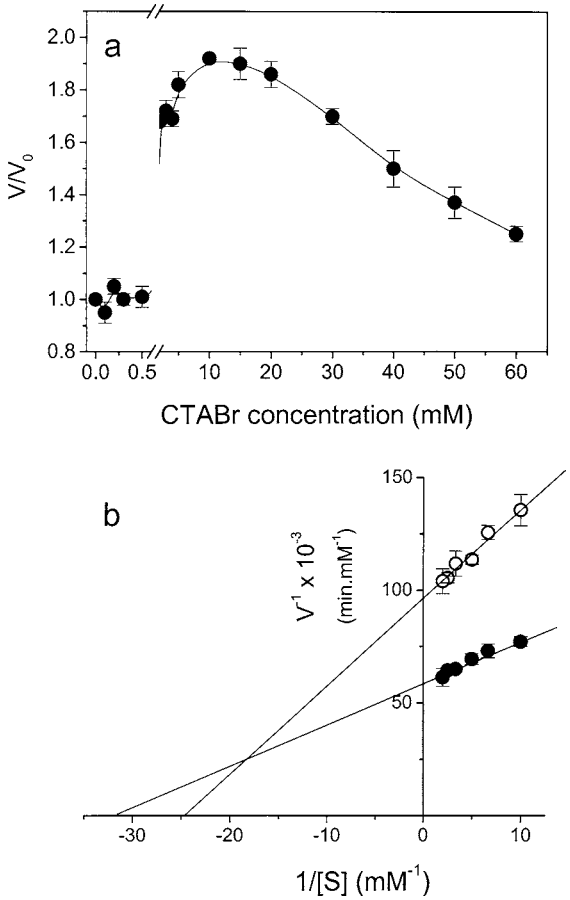

Figure 1 Effect of CTABr on kinetic properties of α-CT

(**a**) The activity of 10 μM α-CT was determined at 25 °C by the rate of α-CT-catalysed hydrolysis of PNPA in 20 mM Tris/HCl, pH 7.5, after 30 min incubation with CTABr at different concentrations. The relative activity (V/V_0) was calculated as a ratio to the original activity (V_0), obtained in the absence of CTABr. The PNPA concentration in the reaction medium was 2 mM. (**b**) Lineweaver–Burk plots for of α-CT activity as a function of initial PNPA concentration, in the absence (○) and presence (●) of 20 mM CTABr.

dependence on CTABr concentration, and at 60 mM CTABr the enzyme activity was about 25 % higher than that found in buffer.

Considering two populations of enzyme, free and micelle-bound, there are four possibilities for catalysis: the free enzyme reacting with (i) free and (ii) micelle-bound substrate, and the bound enzyme reacting with (iii) free and (iv) bound substrate [15]. If no enzyme–micelle interaction takes place, monotonic behaviour of the activity as a function of surfactant concentration is observed. However, if there is an enzyme–micelle interaction, a bell-shaped curve is observed for the activity as a function of surfactant concentration. The model that predicts this behaviour depends on the catalytic efficiency of both the bound and free enzyme [15].

Therefore the bell-shaped profile for α-CT activity as a function of CTABr in concentrations above the cmc found in this work (Figure 1a) indicates an enzyme–micelle interaction. Our data resemble the situation where the substrate is segregated by the surfactant while the bound enzyme reacts with the free substrate [15].

The decrease in activity observed with higher CTABr concentrations can be explained by the unfavourable partition of the substrate into the micelles. Due to an increase in the number of

micelles in solution, there is a decrease in the concentration of free substrate. Since the enzyme reacts with free substrate, the unfavourable partition leads to a decrease in activity when the concentration of surfactant is increased.

In addition, in order to assure that the decrease in activity observed in Figure 1(a) was not due to enzyme inactivation, the stability of α-CT was studied in the absence and presence of CTABr in both monomeric and micellar states. The enzyme is very stable at this temperature, retaining around 85 % (S.D., $\pm$ 1.1 %) of the initial activity after 2 h incubation at 25 °C in buffer only or in the presence of monomeric CTABr. Although the higher enzyme activity could contribute to self-proteolysis, no significant decrease in α-CT stability was found at this temperature. Remaining α-CT activity after 2 h incubation was 86 ± 1.6, 83 ± 3.4 and 79 ± 1.3 % with 10, 20 or 60 mM CTABr respectively. Statistical analysis by Student's *t* test of these results showed that they are not significantly different from that obtained in buffer only. Therefore, even with 2 h incubation with the micelles, the degree of enzyme activation was essentially the same as in Figure 1(a).

The effect of CTABr micelles on the apparent Michaelis constant (K_m^{app}) for PNPA and the catalytic activity (k_{cat}) was also studied. In buffer only, we found that α-CT-catalysed hydrolysis of PNPA showed a K_m^{app} of 44.0 μM and k_{cat} of 1.03 min^{-1} (Figure 1b). In the presence of 20 mM CTABr, there was a decrease in K_m^{app} to 29.7 μM together with an increase in k_{cat} to 1.69 min^{-1}, indicating that the micelle-bound α-CT presents 2.5 times higher catalytic efficiency than the free enzyme.

Nevertheless, Spreti et al. [18] reported an opposite effect with CTABr micelles, where an approx. 20 % decrease was observed compared with the original α-CT activity. However, different substrates were used by these authors (*N*-glutaryl-L-phenylalanine *p*-nitroanilide, GpNA) compared with the present study, and it is reasonable to expect different interactions with the micelle interface. Indeed, the substrate-association constants for CTABr micelles are quite different: $K_S^{CTABr-GpNA} = 2000\ M^{-1}$ [18] and $K_S^{CTABr-PNPA} = 56\ M^{-1}$ (the present study). The $K_S^{CTABr-PNPA}$ value determined here is in a good agreement with previously determined values [14]. It is reasonable to expect that the substrate distribution between the aqueous phase and the micelle interface affects the observed rate of the enzyme-catalysed reaction. Taking into account that the activation behaviour indicates that the bound α-CT reacts with the free substrate, higher K_S values lead to lower concentrations of the free substrate in the water pseudo-phase, and consequently to a decreased enzyme activity with increasing surfactant concentration.

Other studies showed different effects of reversed [6] or aqueous [20] micelles on the kinetic properties of α-CT. A decrease in both the catalytic activity and K_m was related to changes in enzyme conformation. However, although an increased affinity on α-CT was observed with KCl, it occurred without significant changes in enzyme conformation [20]. Furthermore, Spreti et al. [27] reported that the α-CT conformation is affected by a non-micellizable quaternary ammonium salt in concentrations that cause a decrease in both the activity and catalytic efficiency of the enzyme. In order to examine if α-CT activation was related to conformational changes in the protein, we used different spectroscopic techniques to address both the tertiary and secondary structure.

Changes in α-CT tertiary structure

The tertiary structure of α-CT was evaluated by Trp intrinsic fluorescence and near-UV CD spectroscopies. This enzyme presents eight Trp residues distributed on the surface as well as in the core region of the protein [28]. Both the Trp fluorescence emission maximum and the intensity of α-CT spectrum remained unchanged with CTABr monomers (results not shown). However, CTABr micelles caused a significant increase in the intensity of α-CT fluorescence (Figure 2), with a red shift from 334 to 343 nm indicating that some Trp residues are more exposed to the polar medium. The changes both in intensity and in the emission maximum were slow, and stabilized around 10–15 min incubation of the enzyme with the micelles. After that, no significant changes were observed in all spectra recorded up to 2 h incubation.

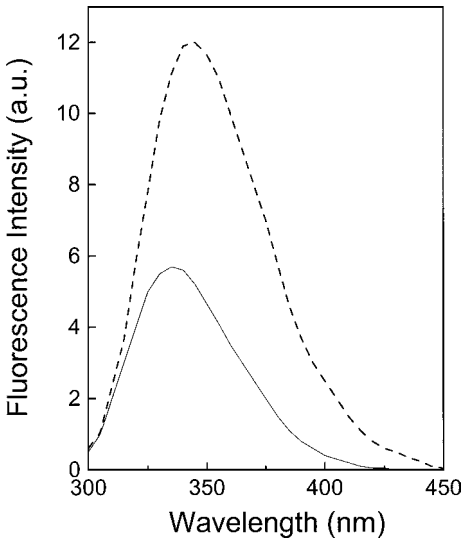

Figure 2 Fluorescence spectra of α-CT with CTABr micelles

The intrinsic fluorescence spectra of α-CT (10 μM) were obtained at 25 °C in 20 mM Tris/HCl, pH 7.5, in the absence (solid line) and presence (dashed line) of 2.0 mM CTABr for 30 min incubation. The samples were excited at 295 nm. Similar spectra were obtained at 2 h incubation, with 2.0, 10.0 and 30.0 mM CTABr. a.u., arbitrary units.

The spectra of 10 μM α-CT recorded with 2.0, 4.0 or 30.0 mM CTABr were essentially the same (results not shown). The increase in intensity of α-CT fluorescence spectrum suggests that in the original conformation at least some of the Trp residues were located close to other residues, such as Arg for instance, which caused internal quenching. Therefore, the interaction with CTABr micelles results in some conformational change where the internal quenching effect can be suppressed.

Most of the Trp residues on α-CT present a low accessibility to the solvent (residues 27, 29, 51, 141, 172 and 215), with less than 20 % of their area exposed to the aqueous medium. Only two Trp residues present around 46 % (Trp^{207}) and 49 % (Trp^{237}) of exposed area to the solvent, and the changes in fluorescence intensity on α-CT spectrum could be due to a local effect on these residues when binding to the micelles without the occurrence of a conformational change. This possibility could be ruled out by the fact that the fluorescence spectrum of *N*-acetyl-L-tryptophan amide is not affected by CTABr micelles [16].

In order to assure that the observed effects were related to conformational changes, near-UV CD was also used. In this technique, the spectrum of a protein is essentially a contribution of aromatic residues and disulphide bonds where the bands can be affected by the flexibility and the number of aromatic side chains [29,30]. Although a detailed interpretation of the near-UV CD spectra of proteins is not always possible, this technique can be very useful to study changes on tertiary structures caused by external factors [29,30].

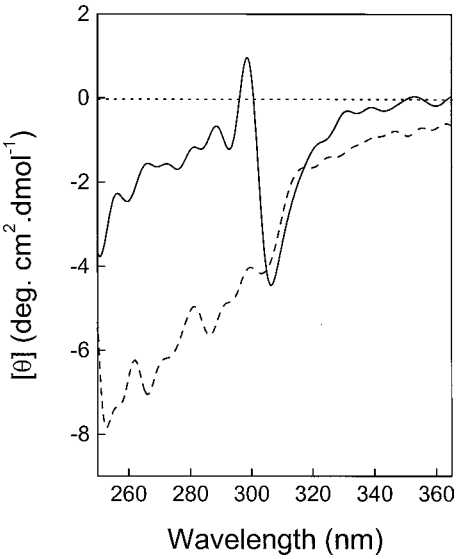

Figure 3 Near-UV CD spectra of α-CT

The near-UV CD spectra of 150 μM α-CT were obtained in 20 mM Tris/HCl, pH 7.5, in the absence (solid line) and presence (dashed line) of 2.0 mM CTABr for 70 min incubation.

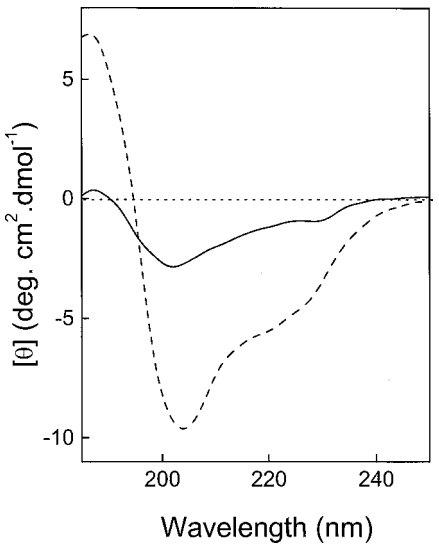

Figure 4 Far-UV CD spectra of α-CT

Far-UV CD spectrum of 20 μM α-CT in 20 mM Tris/HCl, pH 7.5, in the absence (solid line) and in the presence (dashed line) of 2.0 mM CTABr for 30 min incubation. No significant change in the spectrum of α-CT was observed after 2 h of incubation with CTABr.

The near-UV CD spectrum of α-CT (Figure 3) in buffer presents the contributions of Tyr and Trp residues responsible for peaks and shoulders between 270 and 305 nm, and of Phe residues which strongly contribute to bands in the 258–270 nm region [29,30]. After 70 min of incubation with CTABr micelles, significant changes occurred in the 258–305 nm region, indicating a conformational change that leads to a more flexible environment near aromatic residues. Due to a limitation on this technique, that requires high protein concentration, a smaller protein/surfactant molar ratio was used. An increase in the surfactant concentration caused a large increase in spectral noise, and a longer incubation time was needed to achieve equilibration of the sample. Unpublished results from our group (M. G. D'Andrea and M. L. Bianconi) indicate that the kinetics of spectral changes depend on the protein/surfactant molar ratio, being faster as the ratio decreases. In the near-UV CD spectrum of α-CT at 30 min incubation with micelles there is a decrease in the intensity of the positive band at 298 nm while the negative band at 305 nm remained unchanged (results not shown). This result suggests the transfer of Trp residues to a more flexible environment with minor modifications at the vicinities of phenylalanines and tyrosines [29], indicating a substantial change in the tertiary structure between 30 and 70 min incubation with the micelles. These changes would probably be faster with higher CTABr concentrations.

In addition, the intrinsic fluorescence is monitoring only the changes in some of the Trp residues, while the near-UV CD has contributions of all aromatic residues, not only tryptophans. Nevertheless, it is reasonable to assume that the spectral changes observed by intrinsic fluorescence and near-UV CD are related to changes in α-CT conformation upon binding to CTABr micelles.

It has been reported that aqueous micelles of cetyltributylammonium bromide cause an increase in α-CT activity which is accompanied by an increase in intensity as well as a red shift in the intrinsic fluorescence spectrum of the enzyme [18]. Lalitha and Mulimani [17] also found a higher catalytic efficiency for potato acid phosphatase in the presence of low concentrations of CTABr, which occurred with an increase in the fluorescence intensity. These authors suggested that the observed kinetic effects were related to conformational changes in the proteins [17,18]. By using two different techniques, here we show that the interaction with CTABr micelles causes a change in the α-CT tertiary structure.

Changes in α-CT secondary structure

Far-UV CD and FTIR spectroscopies were used as complementary tools for examining the effect of CTABr micelles on α-CT secondary structure. The proportion of the different secondary structures was found by curve fitting of experimental data.

α-CT is folded into two domains with very little α-helix content and extensive regions of anti-parallel β-sheets, having two interchain and three intrachain disulphide bonds [31]. In the conditions used here, the far-UV CD spectrum of α-CT showed a negative band in the 230 nm region (Figure 4) that is related to the catalytic active conformation of α-CT and results from changes in excitation coupling of Trp^{172} and Trp^{215} after activation of the zymogen [29,32].

The far-UV CD spectrum of α-CT in buffer (Figure 4) is also characterized by a minimum at 205 nm and a low-intensity positive band at 188 nm. The anti-parallel pleated sheets on the crystal structure of α-CT are either highly distorted or form very short irregular strands [31], causing the shift of the negative band on the far-UV CD spectrum of α-CT from the expected β-sheet position (210–220 nm) towards the 200 nm region [33]. Spectral deconvolution of α-CT spectrum in buffer showed 8% of α-helix, 30% of β structures, 28% of turns and 34% of unordered structure, very similar to previously reported values [34,35]. No significant changes in the α-CT spectrum were observed in the presence of monomeric CTABr (results not shown). This is in agreement with the kinetic studies where the activity of α-CT was essentially the same in buffer only and with monomeric surfactant.

Nevertheless, the interaction with CTABr micelles caused significant changes in the secondary structure of α-CT (Figure 4). In addition to an increase in intensity, spectral deconvolution of micelle-bound α-CT spectrum showed an increase in the α-helix content to 12% and a decrease in the β-sheet to 26%, while the turns and unordered contents remained unchanged as

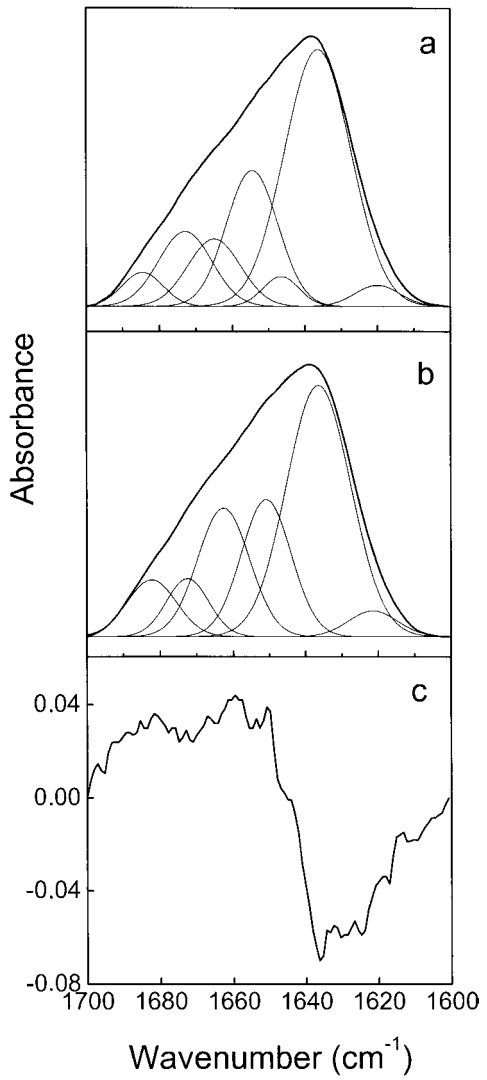

Figure 5 FTIR absorbance spectra of α-CT

(**a**) FTIR absorbance spectra of 267 μM α-CT in 20 mM Tris/HCl buffer, where Gaussian curves are the spectral components obtained by curve fitting. (**b**) As in (**a**) for α-CT in the presence of 3 mM CTABr micelles, taken after 2 h equilibration. (**c**) Difference spectrum obtained by subtracting a normalized spectrum of free protein from a normalized spectrum of micelle-bound protein ($\times$ 100). Similar results were found with 30.0 mM CTABr.

compared with the free enzyme. The far-UV CD spectra of α-CT with 20 mM CTABr presented a high noise to signal ratio. Although spectral deconvolution was not possible in this case, the increase in intensity of the negative peak at 222 nm also suggests an increase in the α-helical content of the protein.

The FTIR absorbance spectra of α-CT in the absence and in the presence of CTABr micelles showed small but reproducible differences (Figures 5a and 5b). The band-fitting analysis and band assignment are summarized in Table 1 and the secondary-structure change was confirmed by the difference spectrum presented in Figure 5(c).

Seven component bands were resolved in the free α-CT spectrum (Figure 5a and Table 1). The maximum absorption was centred at 1637 cm^{-1}, as expected for a protein with a high content of anti-parallel β-sheet. The two bands at 1622 and 1673 cm^{-1} can be attributed to low- and high-frequency components of β-sheet, whereas the band centred at 1655 cm^{-1} corresponds to α-helix conformation. The component at 1647 cm^{-1} is assigned

Table 1 Band position (cm^{-1}) and relative intensities (% Area) obtained from fitting the amide I′ region of the FTIR spectra of α-CT in solution and with CTABr micelles

	α-CT		α-CT + CTABr micelles	
Band assignment	Band position (cm^{-1})	% Area	Band position (cm^{-1})	% Area
β-Sheet	1621.6	4.4	1620.6	3.5
β-Sheet	1636.5	45.5	1635.7	41.2
Unordered	1647.0	2.5		
α-Helix	1654.8	25.8	1652.3	30.8
β-Turns/bends	1665.4	5.3	1666.0	10.9
β-Sheet	1672.7	12.5	1674.4	9.2
β-Turns/bends	1684.6	4.0	1684.5	4.4

to unordered segments, and the bands at 1665 and 1685 cm^{-1} are attributed to β-turns [36]. The resolved components and the determined 62 % β-sheet content are in agreement with previous IR studies of α-CT [36].

Upon binding to CTABr micelles, the main component centred at 1637 cm^{-1}, assigned to β-sheet, was still present (Figure 5b). Although the secondary structure of α-CT is mainly retained (Table 1), there was a reduction in β-sheet content and the disappearance of the component corresponding to unordered structure. The decrease in β-sheet was accompanied by an increase in the bands corresponding to α-helix and turns. These changes were also illustrated by the difference spectrum shown in Figure 5(c), where the normalized spectrum of free protein was subtracted from that of micelle-bound protein. The positive band above 1650 cm^{-1} indicates a higher content of α-helix and turns structures on bound α-CT, and the negative band below 1650 cm^{-1} suggests that the interaction with micelles reduces the unordered and β-sheet content.

In general, although similar results should be expected by using CD or IR spectroscopies, the quantitative absolute secondary structure for α-CT in buffer displayed significant variations when determined by either technique. We have ruled out the possibility of massive aggregation in the FTIR experiments due to the high protein concentration by the absence of prominent bands centred at 1615 and 1685 cm^{-1} characteristic of intermolecular contacts. These bands were observed in self-associated peptides [37], thermally denatured proteins [38] and lipid-associated proteins [39]. Even though soluble aggregates may be still present, our results are comparable with published data with these techniques.

There could be several reasons for this discrepancy. Far-UV CD spectra may be influenced by aromatic residues and disulphide-bond contributions as well as the length and regularity of structural elements [30]. On the other hand, helix bending, the number of adjacent strands in a β-sheet and side-chain absorption in the amide I′ region, among others, could be the source of error in analysing secondary structure by IR spectroscopy [40–43].

The different approaches applied for analysing both CD and IR spectra can also lead to discrepancies in the results. In the former, a method based on the correlation with a reference set of proteins was used. In IR, decomposition of the amide I′ band into components related to different kinds of secondary structure was performed by Fourier self-deconvolution followed by curve fitting. In addition, variations in the secondary-structure content of α-CT determined by far-UV CD have been reported by different authors. While Provencher and Glöckner [34] and Sreerama and Woody [35] found around 30 % of β-sheet on α-CT, the β content detected by Simon et al. [33] was 47 %.

Despite the limitations in assessing quantitative secondary structure, both techniques are useful tools for monitoring changes

in the conformations of the protein backbone in relative terms. Therefore, it is important to note that both CD and FTIR spectroscopy revealed the same trend in α-CT conformational change upon CTABr micelles binding: an increase in the α-helix content together with a reduction in β structure.

Final remarks

The activation of micelle-bound α-CT seems to be related to two different features: the nature of the substrate and the changes in enzyme conformation. Our data indicate that the catalytic behaviour of micelle-bound α-CT is a consequence of the new conformation adopted by the enzyme. However, the low K_S for the PNPA association to the CTABr micelles is an important factor that determines the observed effects, since, apparently, the micelle-bound α-CT preferentially reacts with the free substrate. The substrate distribution between aqueous and micellar pseudophases can be critical for the kinetic behaviour of a micelle-bound enzyme. This distribution will differentiate between an increase and a decrease in the activity if the enzyme reacts with either species (free or micelle-bound substrate). This is evident when comparing our results with those from Spreti et al. [18] that used another substrate with much higher affinity for CTABr micelles, leading to a decreased α-CT activity.

Concerning the conformational changes, the limitations on CD spectroscopy makes it difficult to obtain the α-CT spectra in the same conditions where maximum activity was achieved. However, even though the far-UV CD at this condition could not be deconvoluted, due to the high noise/signal ratio, it showed an increase in the intensity at 222 nm indicating a similar trend of increased α-helix as observed with low micelle concentration.

The intrinsic fluorescence spectrum of the enzyme after incubation with 20 mM CTABr was similar to that found with 2 mM surfactant, although the enzyme is more active in the first condition. However, the intrinsic fluorescence is a measurement of the average emission of the eight Trp residues distributed on the surface as well as in the core region of the protein. It is very possible that small differences in α-CT conformation occur by increasing the micellar concentration, but that they are not being detected by fluorescence.

Nevertheless, as stated above, our data show that the increase in catalytic efficiency is related not only to the distribution of enzyme and substrate between the aqueous and micellar pseudophases, but also to changes in the protein conformation. It is possible that just local changes on α-CT conformation are taking place, but at the tertiary-structural level both fluorescence and near-UV CD indicate that the environment of some aromatic residues is being affected by the interfacial binding. At the secondary-structural level, an increase in α-helical content and a decrease in β structures were observed by far-UV CD and FTIR spectroscopy.

In short, the effects of micellar catalysis indicate that the kinetic behaviour of biological enzymes in the living cell is rather complex and certainly different from that determined in a simple system, i.e. the commonly used diluted solutions with very low concentrations of the particular enzyme and substrates or co-factors.

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