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α-Chymotrypsin superactivity in aqueous solutions of cationic surfactants

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Abstract

α-Chymotrypsin (α-CT) activity was tested in aqueous media with the following cetyltrialkylammonium bromide surfactants in the series methyl, ethyl, propyl and butyl, different in the head group size, and for the sake of comparison also with the anionic sodium *n*-dodecyl sulfate and the zwitterionic myristyldimethylammonium propanesulfonate. *N*-glutaryl-L-phenylalanine *p*-nitroanilide hydrolysis rate was monitored at surfactant concentration above the critical micellar one. Only some cationic surfactants gave superactivity and the head group size had a major weight. The highest superactivity was measured in the presence of cetyltributylammonium bromide. The effect of both nature and concentration of three different buffers was also investigated. There is a dependence of enzyme superactivity on buffer type. Michaelis–Menten kinetics were found. The binding constants of substrate with micellar aggregates were determined in the used buffers and the effective improvement of reaction rate (at the same free substrate concentration in the medium) was calculated. k_{cat} significantly increased while K_m was little changed after correction to free substrate concentration. The ratio of k_{cat} to K_m was between 12 and 35 times higher than in pure buffer, depending on buffer and surfactant concentrations. The increase of α-CT activity (30%) was less important in the presence of 1×10^{-2} M tetrabutylammonium bromide, a very hydrophobic salt, unable to micellise. Fluorescence spectra showed differences of enzyme conformation in the presence of various surfactants.

Keywords: α-Chymotrypsin; Superactivity; Stability; Cationic surfactants; Micellar aggregates

1. Introduction

Surfactant molecules have been largely employed in the past decade in a variety of reverse micelle applications such as surfactant microemulsions [1,2] for extraction of active enzymes [3–6] and preparation of media for hosting enzymatic reactions [7–10]. On the contrary, biocatalysis in buffer–surfactant aqueous solutions was poorly studied even though protein–surfactant systems are useful models for studying interactions between membrane proteins and lipids [11,12]. Reports concerning the effect of surfactant on enzyme kinetics have appeared in the literature but these studies regarded only a small number of systems and

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generally they explored only commercially available surfactants. The most commonly used surfactant in enzymology is the sodium *n*-dodecyl sulfate (SDS) for its denaturing effect in SDS–gel electrophoresis [13]. In addition, the cationic surfactant cetyltrimethylammonium bromide (CTABr) and the anionic double-tailed surfactant sodium bis(2-ethylhexyl)sulfosuccinate (AOT) and various nonionic surfactants among the poly(ethylene glycol) derivatives of the Brij, Tween and Triton series have been frequently employed. It is generally reported that surfactants cause protein denaturation, induce inhibiting effects on enzyme kinetics and only seldom stimulating effects on enzyme activity [1].

Kinetic parameters in ternary systems (organic solvent–surfactant–aqueous buffer) are generally comparable to those in aqueous solutions [1,7,14,15] and derivable from Michaelis–Menten kinetics, but not rarely enzymes exhibited an higher activity than the one expressed in the reference buffer, giving rise to the concept of superactivity [1,2,16–20]. Theoretical models to explain how reverse micelles affect the kinetics [15,21,22] assume that superactivity of hydrophilic enzymes dissolved in the aqueous core of the droplets is due to the interactions of the droplet interface with the molecules and/or to the relatively high rigidity of the enzyme molecule caused by the surfactant layer and/or a higher reactivity of the structured water in the micelle [2,23].

In this literature scenario only few studies have been devoted to investigate rather specific interactions which occur between surfactant and proteins in aqueous solutions and to determine enzyme stability and activity. Schoemaecker et al. [24] studied the interaction of four surfactants (i.e., anionic, neutral, cationic single chain and anionic double-chain) with α-chymotrypsin and various lipases. No correlations were found between lipase behaviour in surfactant aqueous solutions and in reverse micelles. In contrast, correlation of the inhibiting and denaturing effect of some surfactants on α-chymotrypsin was found in the two systems. Creagh et al. [25] studying the structure and catalytic characteristics of alcohol dehydrogenase in aqueous solutions of surfactants underlined the importance of electrostatic and hydrophobic interactions between enzyme and surfactant when designing a reverse micellar system.

The present study was undertaken to investigate the relationships between the chemical structure of synthetic surfactants and their degree of self organization with the activity and stability of a model enzyme in aqueous systems since, in our opinion, an understanding of the factors which could enhance or hinder enzyme activity in the presence of self organizing amphiphilic systems may be valuable. α-Chymotrypsin (EC 3.4.21.1) was selected as model enzyme since it is a widely studied serine protease, its mechanism of action in aqueous media is well known [26] and enzyme superactivity has been well documented with different substrates in reverse micelles [16,17,19,27–30].

Special emphasis has been given in this investigation to the occurrence of α-chymotrypsin superactivity in buffer–surfactant solutions. To meet this end different synthetic ionic and zwitterionic surfactants have been screened and tested at concentration above the c.m.c. (critical micelle concentration) in the enzymatic hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide(GPNA). The selected surfactants were: sodium *n*-dodecyl sulfate, SDS; myristyldimethylammonium propanesulfonate, SB3-14; cetyltrimethylammonium bromide, CTABr; cetyltriethylammonium bromide, CTEABr; cetyltripropylammonium bromide, CTPABr and cetyltributylammonium bromide, CTBABr. They differ in head group charge and size in the homologous series of the cethyltrialkylammoniun bromide thus providing a distinct environment that alters both electrostatic and hydrophobic interactions and should play a key role in determining the enzyme catalytic performance [31]. The ammonium salt, tetrabutylammonium bromide, TBABr, was also tested in the attempt to discriminate among the role played by the

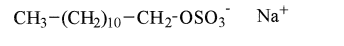
head group and the alkyl tail of the cationic surfactant.

The effect of those surfactants, which induce superactivity, was compared in different environments prepared varying the buffer species and their concentrations. The evaluation of the kinetic parameters together with a discussion of the hypotheses which could support the observed α-chymotrypsin superactivity is also reported.

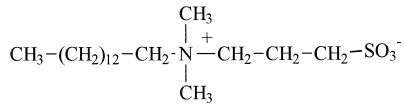
2. Experimental

α-Chymotrypsin (α-CT) from bovine pancreas (molecular weight 24.8 kDa, isoelectric point pI 8.8) was purchased from Sigma (USA) and used without further purification. α-CT was Type II Sigma preparation, 3 times crystallised, dialysed, and lyophilised. The substrate, *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA), was also supplied by Sigma. Enzyme and substrate solutions were always freshly prepared in the appropriate buffer immediately before their use in experiments. The chemicals used for buffer preparation were: Tris(hydroxymethyl)-aminomethane (TRIS) (pK_a 8.3) from Aldrich (Germany), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) (pK_a 7.35) from Sigma, mono and bibasic potassium phosphate (pK_a 6.82) from Panreac (Spain). All other chemicals were of analytical grade. The commercial grade surfactant, SDS, SB3-14 and CTABr were from Fluka (Germany), TBABr was from Aldrich. They were purified as reported in Refs. [31–34]. The preparation and purification at laboratory scale of the synthesised surfactants, CTEABr, CTPABr and CTBABr have been fully described in Ref. [35]. All surfactants were chemically pure as tested by elemental analysis. Furthermore, minima were not present in surface tension versus surfactant concentration plots thus assuring the absence of any hydrophobic impurities. Table 1 lists the different tested surfactants along with their chemical formula and abbreviations.

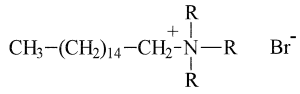
Table 1
Structure of the surfactants used in this work



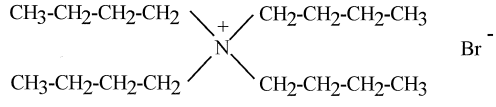
Sodium n-dodecyl sulfate (SDS)



Myristyldimethylammonium propanesulfonate (SB3-14)



R=CH_3; Cetyltrimethylammonium bromide (CTABr)
R=CH_2CH_3; Cetyltriethylammonium bromide (CTEABr)
R=$CH_2CH_2CH_3$; Cetyltripropylammonium bromide (CTPABr)
R=$CH_2CH_2CH_2CH_3$; Cetyltributylammonium bromide (CTBABr)



Tetrabutylammonium bromide (TBABr)

2.1. Assay of α-chymotrypsin activity

The α-chymotrypsin catalysed hydrolysis of GPNA was monitored by following the change in absorbance at 410 nm due to the formation of *p*-nitroaniline (PNA). Kinetic determinations were performed at 25.0°C using a Shimadzu UV-160A UV-VIS spectrophotometer equipped with thermostated cell holders controlled at ±0.1°C. The product extinction coefficient was 8750 m^{-1} cm^{-1} either in pure buffer or in the presence of surfactants. The autohydrolysis of GPNA (PNA formation without enzyme in the cuvette) was not detected during the time-scale of the experiments (initial 10 min) at all the explored conditions. Enzyme activity was typically assayed in 0.1 M buffer pH 7.75 (unless

otherwise specified) and with 2.5×10^{-3} M GPNA. α-Chymotrypsin concentration was 8 μm (0.2 mg ml^{-1}).

Kinetic tests were regularly carried out in a 3 ml cuvette with 1 cm pathlength, filled with surfactant and substrate solutions both prepared in buffer. The enzymatic reaction was started by addition of 60 μL of the enzyme buffered stock solution (10 mg ml^{-1}).

The α-CT activity was evaluated either as specific initial reaction rate r, defined as moles of PNA formed per unit weight of enzyme (mg) and second, or as turnover number, k_{cat} (s^{-1}), moles of GPNA transformed per second and per mole of enzyme.

In all the experiments, PNA formation during the very first minutes of hydrolysis at saturating substrate concentration was linearly time dependent and the rate was calculated from the slope of changes in absorbance versus time records. The values were related to the overall enzyme amount to represent α-CT activity. Rate constant, V_{max}, and Michaelis constant, K_m, in the presence of buffer and/or surfactant were obtained by linear regression analysis of the double reciprocal Lineweaver–Burk plot and the k_{cat} value was calculated.

All sets of experiments were reproduced several times under identical operating conditions in order to increase the accuracy of the findings and each data point of a set of results was obtained at least in duplicate and the discrepancy was below 5%.

2.2. Enzyme stability in the presence of the surfactant

Stability of α-CT was studied by incubating solutions of the enzyme (1 mg/ml) at 25°C in pure buffer or in buffer plus surfactant. Samples were periodically withdrawn and the residual activity was measured in the presence of saturating GPNA concentration (2.5×10^{-3} M). No deactivation of the enzyme in all the different solutions was detected during the short-times (10 min) used for kinetic studies.

2.3. Determination of critical micelle concentration (c.m.c.)

The c.m.c. of surfactants were determined at 25°C by measuring the surface tension of the solution containing either buffer and enzyme at different surfactant concentrations, by using a Krüss du Nouy type tensiometer, according to the standard procedure reported in the literature [36].

2.4. Determination of the binding constant (K_s)

Assuming GPNA partially bound to surfactant micelles, the material balance for the substrate gives:

$$[S_T] = [S_W] + [S_M] \quad (1)$$

where S_T stands for concentration of analytical substrate, S_W for free substrate one and S_M is that retained by aggregates.

The following equilibrium was assumed:

$$[S_W] + [D_n] \rightleftarrows [S_M] \quad (2)$$

where D_n, the micellised surfactant concentration, was calculated from the difference between the total surfactant added to the system and the c.m.c. The binding constant K_S is related to surfactant and substrate concentration by the following equation:

$$K_S = \frac{[S_T] - [S_W]}{[S_W][D_n]} \quad (3)$$

K_S values were calculated with a least square fit of Eq. (4) [37]:

$$A_\lambda = \frac{(\varepsilon_W + \varepsilon_M K_S[D_n])}{1 + K_S[D_n]}[S_T] \quad (4)$$

where ε_W and ε_M are the molar extinction coefficients of free and bound GPNA respectively. They were evaluated measuring $A_{328\ nm}$ of GPNA from experimental tests at buffer concentrations in the range 0.1–0.4 M. The following values for ε_W in buffer were determined: 11,500 in TRIS–HCl; 11,900 in HEPES and 11,500 in phosphate. The ε_M determined in

buffer at the highest concentration of surfactant were: 15,000 in TRIS–HCl; 14,200 in HEPES and 14,400 in phosphate. K_S was not dependent on buffer molarity.

2.5. Fluorescence studies

Fluorescence experiments were performed on a Perkin-Elmer LS-50-B spectrofluorimeter with a 1 cm cuvette. The α-CT samples were excited at 295 nm and fluorescence spectra were registered from 310 to 380 nm. Intensity was corrected for absorbance of the solutions without the enzyme. α-Chymotrypsin solutions (8 μM) were prepared either in pure buffer 0.1 M TRIS–HCl buffer pH 7.75 or in surfactant-aqueous buffer medium (CTBABr 5×10^{-3} M or SDS 1×10^{-3} M). Because of limits in the linearity of intensity readings the enzyme solutions were diluted with buffer in a 1:20 ratio in the fluorescence cell. The ratio of surfactant molarity to enzyme concentration was the same as in the kinetic study and surfactant concentration above the c.m.c. was assured.

3. Results and discussion

3.1. Effect of surfactant head group charge and size

This study was initially oriented to establish the effect of charge and size of surfactant head group on α-CT activity in TRIS–HCl buffer. Conditions for GPNA hydrolysis were chosen close to those currently described in the literature for comparative purposes [29]. The pH of aqueous phase (7.75) used for the preparation of protein solution directly affects the protein net charge. Being this pH-value below the α-chymotrypsin isoelectric point (8.8) the enzyme has a net positive charge. Substrate concentration (2.5×10^{-3} M), was higher than the saturating substrate one in pure buffer, and surfactant concentration (1×10^{-2} M), well above the c.m.c., was used in all the experiments. The results expressed as the ratio of specific hydrolysis rate in the presence of surfactant and buffer (r_{sb}) to that in pure buffer (r_b) are quoted in Table 2. GPNA hydrolysis was also run in the presence of surfactant but without enzyme, since many hydrolytic reactions are influenced by the presence of micellar aggregates [38–40]. Micellar catalysis was not effective in these experimental conditions, as no product formation was observed during the time interval usually adopted for the experiments. Inspection of the data in Table 2 shows that the α-CT activity strongly depended on the structure and charge of the head group. The anionic surfactant, SDS, lowered the specific rate of enzymatic hydrolysis by a factor of 8. The inactivation was less important in the presence of the zwitterionic surfactant, SB3-14, the observed activity loss being only 25%. In the presence of cetyltrialkylammonium bromide (cationic) surfactants the GPNA hydrolysis rate depended on the head group size. In comparison with the value in pure buffer the GPNA hydrolysis rate was halved in the presence of both CTABr and CTEABr while was two fold higher and 5.9 times higher by using CTPABr and CTBABr, respectively. This allow to state that large superactivity does not involve only micelle formation. The body of the above results indicate that specific interactions between the cationic surfactant and the enzyme should be effective in determining the α-CT superactivity as the hydrophobicity together with the size of the head groups are increased. This result may be attributed to the progressive in-

Table 2
Ratio of GPNA specific hydrolysis rate with different surfactants

Surfactant	r_{sb} / r_b
SDS	0.12
SB3-14	0.75
CTABr	0.52
CTEABr	0.54
CTPABr	2.04
CTBABr	5.88
TBABr	1.30

[Surfactant] = 1×10^{-2} M; [TRIS–HCl] = 0.1 M, pH 7.75; [GPNA] = 2.5×10^{-3} M; [α-CT] = 8 μM.

crease of microinterface net charge [41,42] since affinity of surfactant monomers and aggregates for counterions decreases with increasing bulk hydrophobicity of alkyl head groups in the series methyl < ethyl < *n*-propyl < *n*-butyl. Besides, it is well known that head group enlargement from CTABr to CTBABr dramatically changes the degree of ionization (α) in micellar aggregates, i.e., α of CTABr is approximately 0.2 and for CTBABr α is ca. 0.5 [35]. This is also in agreement with the formation of weaker ion pairing for monomeric surfactants [43].

Experiment carried out by using TBABr, as a cationic hydrophobic salt unable to micellise, showed only a 30% increase of enzyme activity as reported in Table 2. Other mechanisms, where both electrostatic and hydrophobic interactions play a role, should be also important.

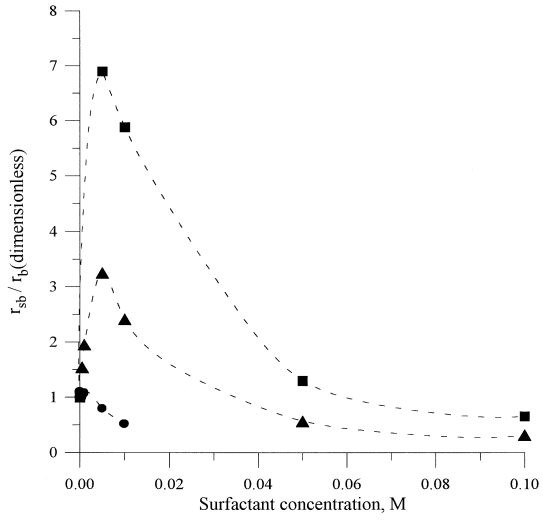


Fig. 1. Effect of surfactant concentration on α-chymotrypsin activity in 0.1 M TRIS–HCl buffer, pH 7.75 at 25.0°C; [GPNA] = 2.5×10^{-3} M; [E] = 8 μM. (●) CTABr, (▲) CTPABr, (■) CTBABr.

3.2. Effect of surfactant concentration and buffer nature

The effect of surfactant concentration on α-CT activity was mainly investigated with those chemicals which provided superactivity. Experiments were carried out in 0.1 M TRIS–HCl buffer, pH 7.75 and at CTPABr and CTBABr concentration from 1×10^{-4} to 1×10^{-1} M. CTABr concentration was below 1×10^{-2} M being it insoluble at higher concentrations. The detected r_{sb} to r_b ratio was reported in Fig. 1 as function of total surfactant concentration in the medium and depended on both the type and the concentration of surfactant. GPNA hydrolysis rate was not dependent on CTABr concentration in the range 1×10^{-4}– 1×10^{-3} M whereas at higher surfactant concentration (1×10^{-2} M) was slightly depressed. Enzyme behaviour was similar in the presence of CTPABr and CTBABr. The highest increase of enzyme superactivity occurred at surfactant concentration equal to 5×10^{-3} M and was annihilated ($r_{sb}/r_b = 1$) in CTPABr 3×10^{-2} M and in CTBABr 5×10^{-2} M.

Surfactants acting as salts in solution may differently interact with the buffer species and alter the pH in the vicinity of reaction site. Consequently, buffers with almost the same pK_a may also have distinct interactions with microinterfaces. The hydrophilic buffers will be more favourable partitioned towards water phase than the hydrophobic ones. Both effects may modulate the activity of the enzyme, by controlling the local pH and by changing the properties of the microinterfaces. In fact, the apparent pK_a of buffer is controlled by the interactions with the surfactant head group which in our case may stabilise the anionic form and change the concentration of the species according to the mass action low [38]. The study was extended to other two buffers with a quite different chemical structure, phosphate and HEPES. All the used buffers show in water the highest buffering capacity at pH 7.75 but they strongly differ in hydrophobicity. In the absence of surfactant in the reaction medium α-CT kinetics was independent of the used buffer. GPNA hydrolysis was followed either at constant buffer concentration, 0.1 M, but varying CTBABr concentration from 5×10^{-3} M to 0.1 M or at constant surfactant concentration 5×10^{-3} M but vary-

ing buffer concentration from 0.1 to 0.4 M. The results are quoted in Table 3 and in Fig. 2.

The extent of α-CT superactivity significantly depends on the buffer used only at the lowest CTBABr concentration. The measured values, 6.9, 9.0 and 10.7, in TRIS–HCl, HEPES and phosphate buffer clearly prove the large positive effect of surfactant addition, which tends to disappear increasing its concentration (Table 3).

Similar experiments with CTABr concentration from 10^{-4} M to 10^{-2} proved that α-chymotrypsin was not affected by the type of used buffer.

The sensitivity of α-CT activity to buffer concentration, especially phosphate, was reported in Refs. [44,45] and confirmed by data of Fig. 2. In the investigated range GPNA hydrolysis rate varied within 15% in pure TRIS–HCl and HEPES buffers, and within 34% in pure phosphate buffer. On the contrary, reaction rate in the presence of CTBABr was independent of phosphate buffer concentration while it dropped when the concentration of HEPES and TRIS–HCl buffers increased. Phosphate buffer was also the most effective to induce α-CT superactivity varying r_{sb}/r_b values between 10.7 and 7.5 times in the investigated buffer concentration interval. In HEPES solutions superactivity ranged from 9.0 to 5.1 times and in TRIS–HCl buffer from 6.9 to 2.7 times.

The ionic strength in the reaction medium varies at constant analytical pH (7.75) being the pK_a of used buffers slightly different. Consequently, the α-CT superactivity could be also due to a change of microenvironment around the enzyme molecule induced by 'pairing' of the surfactant and the buffer and ultimately be a result of modified protein–surfactant–substrate electrostatic interactions. This can alter the velocity constant because of modulation of enzyme surface charge that brought about a catalytically favorable conformation of the enzyme.

A unique correlation of r_{sb}/r_b in the three buffers versus the ionic strength was tried but the data (not reported) did not fit a single line, thus indicating that α-chymotrypsin superactivity did not depend exclusively on the total ion charge in the solution even though tended to disappear when the ionic concentration increased.

The reduction of reaction rate, with increasing buffer concentration but at constant CTBABr concentration, was attributed to interactions between buffers and surfactant. The charged ions, their size and the hydrophobicity of the used buffers being very different, the buffer charged group could differently or not at

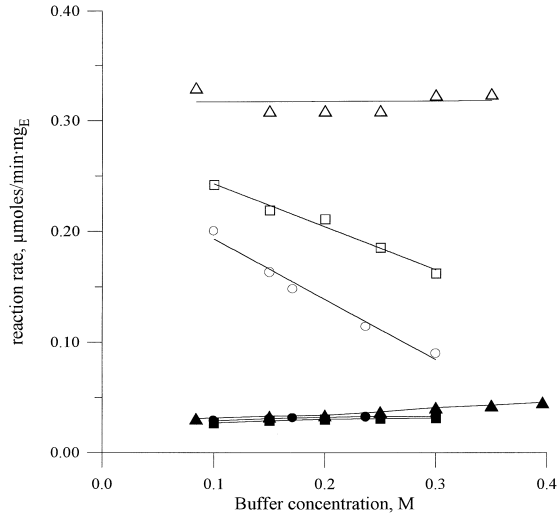


Fig. 2. Effect of buffer concentration on α-chymotrypsin specific activity in pure buffer (closed symbols) and buffer plus 5×10^{-3} M CTBABr (open symbols) at 25.0°C. [GPNA] = 2.5×10^{-3} M; [E] = 8 μM. (●/○) TRIS–HCl, (■/□) HEPES and (▲/△) phosphate buffers.

Table 3
Ratio of GPNA specific hydrolysis rate as a function of CTBABr concentration in different buffers (0.1 M, pH 7.75) at 25.0°C

10^3 [CTBABr] (M)	r_{sb}/r_b		
	HEPES	Phosphate	TRIS–HCl
5.0	8.96	10.65	6.90
10.0	6.00	6.55	5.88
30.0	1.90	2.30	–
50.0	1.22	1.28	1.30
100.0	0.58	0.60	0.66

[GPNA] = 2.5×10^{-3} M; [α-CT] = 8 μM.

all shield the positively charged surfactant head group with a consequent distinction of surfactant-induced enzyme activation. Besides, TRIS–HCl and HEPES also present an apolar part that could interact with the hydrophobic regions of surfactant molecules.

3.3. Role of CTBABr on enzyme kinetics

The effect of surfactant on kinetic parameters was investigated in 0.1 M buffer at substrate concentration from 1.25×10^{-4} M to 5×10^{-3} M and 5×10^{-3} M CTBABr which induced the most important α-chymotrypsin superactivity. Kinetics was also monitored in pure buffer. Independently of the presence of surfactant aggregates in the reaction media data points obey to a Michaelis–Menten kinetics and can be correlated in the Lineweaver–Burk plot for an estimation of the kinetic parameters reported in Table 4. The enzyme behaved similarly in the three pure buffers being both kinetic parameters, k_{cat} and K_m, almost identical. The K_m values determined in surfactant aggregate media were one order of magnitude higher in TRIS–HCl and phosphate and three times higher in HEPES. The k_{cat} values also enormously increased resulting in TRIS–HCl, HEPES and phosphate 13.4, 12.9 and 32.0 times higher than in the same pure buffer respectively. At this point, one could simply state that the catalytic efficiency, i.e., k_{cat}/K_m, in the presence of surfactant aggregates is slightly greater and attribute the observed decrease in substrate affinity (higher K_m) to microenvironment changes nearby the active site caused by the surfactant interactions. However, these kinetic parameters could be also considered apparent since they were evaluated taking into account the analytical substrate concentration. Besides, most of the substrate could partition between the micellar aggregates and the bulk reaction medium. Consequently, nearby the enzyme active site the local substrate concentration would be lowered; thus, K_m would be greatly affected. A similar analysis was made by Ryu et al. [46] who rationalised the increase of K_m to the attractive concept that solvent hydrophobicity affects partitioning of the substrate between solvent and the active site. Indeed, GPNA is only slightly soluble in water and needs for solubilisation to be deprotonated at alkaline pH. Strong interactions between the negatively charged GPNA, due to the free carboxylic group at the amino terminus, and the positively charged surfactant aggregates can be expected and the available free substrate concentration for catalysis could be effectively lower.

Table 4
Kinetic constants for α-chymotrypsin at 25.0°C

	$10^3\ K_m$ (M)	k_{cat} (s^{-1})	k_{cat}/k_m ($M^{-1}\ S^{-1}$)
TRIS–HCl buffer	0.39	1.46×10^{-2}	37.43
HEPES buffer	0.43	1.29×10^{-2}	30.00
Phosphate buffer	0.40	1.38×10^{-2}	34.50
CTBABr in TRIS–HCl buffer	3.69	19.5×10^{-2}	52.85
CTBABr in HEPES buffer	1.42	16.7×10^{-2}	117.61
CTBABr in phosphate buffer	5.89	44.7×10^{-2}	75.89
CTBABr in TRIS–HCl buffer[a]	0.43	19.5×10^{-2}	453.49
CTBABr in HEPES buffer[a]	0.39	16.5×10^{-2}	423.08
CTBABr in phosphate buffer[a]	0.37	44.7×10^{-2}	1208.11

[a] Data after correction to free substrate: K_s(TRIS–HCl) = 1500 M^{-1}; K_s(HEPES) = 500 M^{-1}; K_s (phosphate) = 3000 M^{-1}. [Buffer] = 0.1 M, pH 7.75; [α-CT] = 8 μM; [CTBABr] = 5×10^{-3} M.

In order to validate this hypothesis the binding constant (K_S) of GPNA with CTBABr aggregates was determined at the experimental conditions that gave α-CT superactivity. The values of the binding constants in the three buffers reported as footnote in Table 4 are quite different and confirm the importance of buffer role. The free substrate concentration available for catalysis, $[S_W]$, was calculated by K_S definition, according to Eq. (3). The higher the K_S,

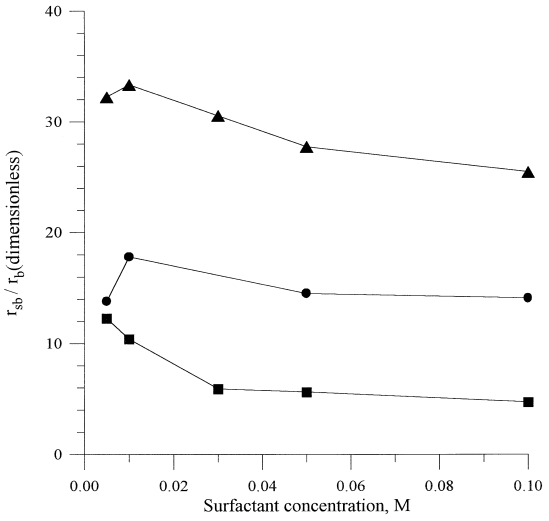


Fig. 3. Effect of CTBABr concentration on α-chymotrypsin superactivity. Data after correction to free substrate. (●) TRIS–HCl, (■) HEPES and (▲) phosphate buffers.

the lower the free substrate concentration, $[S_W]$. On the assumption that the enzyme is present solely in the water phase and is active only towards free substrate molecules the experimentally determined reaction rates were correlated to the effective free substrate concentration $[S_W]$ in the presence of surfactant aggregates.

All data points still fitted the Lineweaver–Burk plot. Regression coefficient was always higher than 0.97. The Michaelis constant, K_m, and the rate constant, k_{cat}, after data correction to free substrate concentration were estimated and listed in Table 4 together with the catalytic efficiency, k_{cat}/K_m. It is interesting to note that k_{cat} values remained unchanged while K_m dropped to values very close to those in pure buffer, that is enzyme affinity for free substrate remained almost unchanged. Consequently, the enzyme efficiency, k_{cat}/K_m, resulted largely increased. The enzyme superactivity cannot be any more related to partitioning of substrate (which has been considered upon K_S correction) but to enzyme specific interactions with the surfactant moiety and microinterfaces.

The data of Table 3 were revised in order to verify the correctness of the previously observed trend. The original r_{sb}/r_b values were calculated from reaction rates in experiments performed at the same overall GPNA bulk concentration (2.5×10^{-3} M) without considering substrate partition to micellar aggregates. The reaction rate in pure buffer was calculated using the Michaelis–Menten kinetic parameters (Table 4) and the effectively available substrate concentration for catalysis, $[S_W]$, from Eq. (3). The r_{sb}/r_b values after correction to free substrate concentration were reported in Fig. 3. α-CT superactivity in CTBABr-buffer media resulted even more evident in all the investigated concentration range and its dependence on surfactant concentration appeared less important.

3.5. Enzyme stability

Fig. 4 depicts the time course of α-CT activity in pure aqueous buffer (TRIS–HCl 0.1 M, pH 7.75) and in this medium containing surfactants as well. The ratio of instantaneous specific reaction rate to that in pure buffer at zero time was reported in the semi-log plot as function of storage time in order to easily compare the deactivation kinetics in the different media and

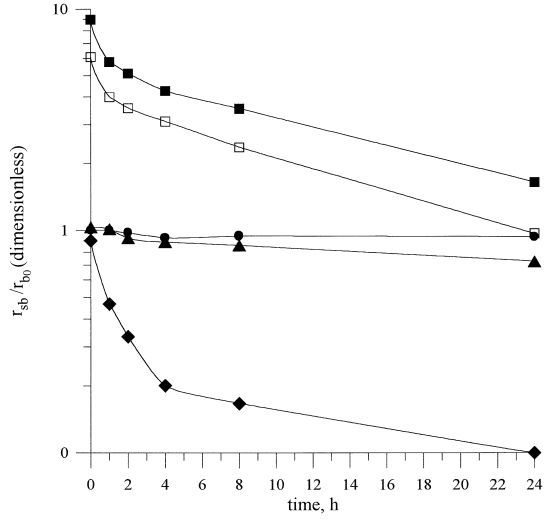


Fig. 4. Surfactant effect on α-chymotrypsin storage stability in TRIS–HCl 0.1 M, pH 7.75 at 25.0°C. (●) no surfactant; (▲) SB3-14 2×10^{-3} M; (◆) CTABr 5×10^{-3} M; (■) CTABBr 5×10^{-3} M and (□) CTBABr 1×10^{-2} M.

to display superactivity preservation with storage time. Stability was monitored at 25°C and the investigated surfactant concentrations are those reported in the caption for figure. The kinetics of irreversible α-CT deactivation follows a simple first-order model (line at constant slope equal to $-k_d$, kinetic constant of the deactivation rate) in pure buffer and in the presence of SB3-14. In the other cases, the mechanism is clearly a complex multistep pattern. Enzyme activity decreased more sharply in the presence of surfactant than in pure buffer. SB3-14 and CTABr which depressed almost at the same extent α-CT activity caused largely different rates of deactivation. CTBABr induced enzyme superactivity and deactivation at a rate intermediate of those caused by the other two surfactants. No direct evidence of correlation between enzyme superactivity and deactivation kinetics arises from these results even though it is quite clear that the surfactant head group plays an important role also in enzyme deactivation. CTBABr concentration did not alter the time course behaviour of enzyme stability. The observed difference in absolute α-CT activity are related to the free substrate available for biocatalysis. Interestingly, the superactivity induced by CTBABr is still preserved during the first 24 h storage, in spite of the faster enzyme inactivation, because of the specific initial reaction rate much higher than that in pure buffer.

The overall observed behaviour could be attributed to alterations of the protein structure in the presence of surfactant which makes the enzyme more sensible to inactivation and possibly more active as it occurs with CTBABr.

3.6. Fluorescence studies

Eight tryptophan residues are present in the α-CT molecule. Two tryptophan residues (Trp-27 and -29), buried in the internal region, are almost inaccessible, while the other residues (Trp-51, -141, -172, -207, -215 and -237) are on the surface of the enzyme molecule [47,48]. Trp-172 and -215, located near the surface of the enzyme, are the most hydrophobic ones, while Trp-27 and -29, buried within the core of the molecule, form hydrogen bonds with internal water molecules and then they are the most hydrophilic ones [49].

For multitryptophan proteins it is difficult to draw quantitative interpretations of fluorescence data but differences in fluorescence spectra can be nevertheless associated with structural modifications of the enzyme.

Fluorescence experiments were performed (see Section 2 for details) at surfactant concentration smaller than the one used in activity and stability runs. However, an independent determination of α-CT kinetics in this condition confirmed that enzyme superactivity is preserved ($r_{sb}/r_b = 5.88$).

Fig. 5 shows the effect of medium composition on fluorescence intensity (I) and wavelength of maximal emission (λ_{max}) for α-CT. The maximal emission was centered at 337 nm in TRIS-HCl buffer. Upon addition of SDS, 1×10^{-2} M, which drastically curtailed enzyme activity, I abruptly decreased from 254 to 132 while λ_{max} grew to 350 nm. The addition of CTBABr, 5×10^{-3} M, which largely promoted enzyme activity, gave the same shift of λ_{max} but

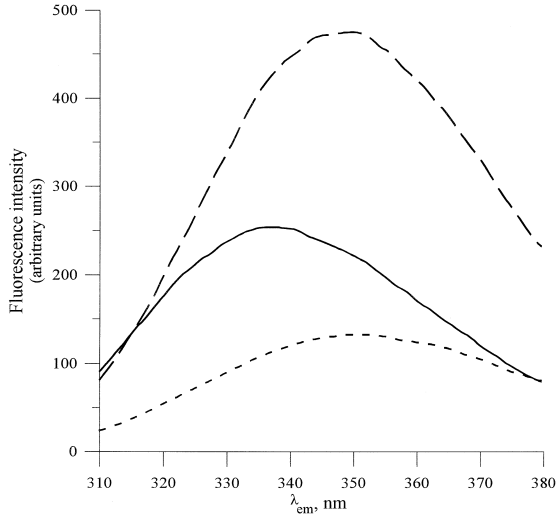


Fig. 5. Fluorescence of α-chymotrypsin in 0.1 M TRIS–HCl buffered solutions, pH 7.75 at 25.0°C. (————) no surfactant; (— — — —) CTBABr 5×10^{-3} M; (---) SDS 1×10^{-2} M.

caused an abrupt increase of fluorescence intensity. These results did not agree with the usually reported evidences. Because of more intensive contact of aromatic chromophores in protein with polar environments, increases in both fluorescence intensity and wavelength of maximal emission are proofs of protein denaturation.

However, as also reported in Ref. [50], changes of I and λ_{max} cannot be caused alone by a trivial influence of medium composition on α-CT fluorescence since it is sensitive to the environment of the fluorophore, to the presence of internal quenching groups and to the energy transfer to other tryptophans [51].

In conclusion, the same emission shifts to a higher wavelength in the presence of SDS and CTBABr indicates an equivalent increase in the polarity of the environment [1,25,52], while the different emission intensity (negative and positive shifts in comparison with values in pure buffer) can be considered an independent proofs of α-CT conformational changes which are responsible for the observed variations of enzyme activity.

4. Conclusions

The experimental results presented in this paper support the following conclusions regarding α-chymotrypsin superactivity aquoeus solution in the presence of a homologous series of cationic surfactants having the same tail length. The role of surfactant cationic head group appears to be particularly important in determining the positive interactions that induce the enzyme superactivity. The increase of the alkyl head group hydrophobicity in the series methyl < ethyl < *n*-propyl < *n*-butyl leads to a marked enhancement of the observed enzyme activity. α-CT superactivity is achieved in buffered media containing surfactant aggregates of both CTPABr and CTBABr. CTBABr is the most effective in determining it. The long hydrophobic tail of surfactant should also play a fundamental role as suggested by the small addition of α-CT activity brought about the non micellising cationic hydrophobic salt, TBABr. The buffer also modulates α-CT superactivity and the phosphate buffer results the most effective.

Because of the unfavourable electrostatic interactions surfactant-enzyme and the favourable one surfactant-substrate, GPNA is largely segregated by surfactant aggregates as shown by the high values of the binding constants of the micellised substrate, while all the enzyme should be available as free protein in solution.

The dependence of enzyme activity either before or after data correction for free substrate concentration on the hydrophobicity of surfactant alkyl head group seems to prove that the hydrophobic interactions between surfactant and enzymes should play a dominant role. Determination of kinetic parameters which consider only the free substrate and all the enzyme proves that the same affinity constant, K_m, is operative in these systems. Consequently, superactivity should be related to a catalytically more favourable conformation of the enzyme.

However, the α-CT superactivity being also modulated by the type of buffer and its concentration the hypothesis was also made that both electrostatic and hydrophobic interactions between buffer and surfactant can alter those of surfactant with enzyme.

Several more experiments are currently in progress to better understand the role of amphiphilic molecules in controlling the properties of biochemical probes and to clarify the mechanism by which enzyme superactivity can be designed.

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