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α-Chymotrypsin superactivity in quaternary ammonium salt solution: kinetic and computational studies

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As previously reported, quaternary ammonium salts with bulky hydrophobic portions provoke a superactivation of α-chymotrypsin in aqueous solution: this is the case of the surfactant cetyltributylammonium bromide (CTBABr) and the corresponding salt tetrabutylammonium bromide (TBABr). In order to achieve a broader knowledge of the enzyme–additive interactions, in this paper the activity and stability of α-chymotrypsin were tested in the presence of additives with slightly modified bulky ammonium groups. The effect of three additives with a benzylic group as substituent (benzyltrimethylammonium bromide (BzTMABr), benzyltributylammonium bromide (BzTBABr) and benzyldodecyldimethylammonium bromide (BzDDABr)) was investigated. A significant increase in instantaneous activity, but a deactivation of enzyme, faster than in pure buffer, was observed. Moreover, two novel dicationic salts, (1,8-bis(tributylammonium)octane dibromide (bisBOAB) and 1,4-bis(tributylammonium)xylene dibromide (bisBAB)) were designed and synthesized in order to evaluate the effect of two tributylammonium head groups with a different spacer. BisBOAB provoked superactivation and stabilization effects in a way similar to the "homologue" TBABr, but at lower concentration. In contrast, when the benzyl group was constrained within the spacer structure, the obtained superactivity was lower than in the presence of a more flexible hydrocarbon chain spacer, and enzyme deactivation was faster. Molecular modelling studies allowed us to rationalize the hypotheses derived from kinetic evidence. The results confirmed that the improvement in the catalytic properties observed in the presence of additives with a bulky, hydrophobic ammonium head group could be addressed to an increase in the overall hydrophobicity of the α-chymotrypsin catalytic site.

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Introduction

The effect of surfactants on enzymatic performance in aqueous solutions has been widely investigated and reviewed.[1–4] Different techniques have been used to study protein–surfactant interactions and the mechanism of protein denaturation by sodium dodecyl sulfate (SDS) has been studied in many different works. The nature of the interaction has been reported to be both electrostatic and hydrophobic. An initial electrostatic binding occurs between the charged head group of the surfactant and ionic sites on the protein surface; this is followed by lipophilic interactions between the alkyl chain of the surfactant and hydrophobic amino acid residues. These interactions seem to cause the unfolding of the enzyme tertiary structure and the loss of its activity.

The presence of surfactants and other additives can also produce conformational changes in the enzyme that allow achieving better catalytic properties. In such cases, functional groups of additives play a relevant role in these effects, since any selective interaction may depend on the structures of both enzyme and the additive.

Furthermore, in aqueous solutions the surfactant can exist as monomers (below the critical micelle concentration, CMC) or as micellar aggregates (above the CMC); so the effect of surfactant on enzyme structure and catalytic properties also depends on its concentration.

The effect of additives on enzymatic activity has been also studied in many relevant works in literature concerning covalent binding of amphiphiles to enzymes. Better catalytic properties of the conjugates were observed both in aqueous solutions[5] and in organic solvents,[6,7] increasing the relevance of using amphiphilic additives in this topic.

α-Chymotrypsin (EC 3.4.21.1) is a globular serine protease that has been widely studied in aqueous solutions, since its structure and mechanism of action are well known.[8–10] Thus it

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was selected as a model enzyme to investigate the effects of differently structured additives on its catalytic properties.

Recently biological buffers, polyelectrolytes and polyamines were used to improve the catalytic activity of α-chymotrypsin (α-CT); favorable electrostatic interactions between the additive and the enzyme, or substrate, seemed to contribute to the superactivation of α-CT.[11–13]

Many works in the literature dealt with the effect of surfactants on α-CT activity; in one of the early papers the inhibiting effect of several ionic and neutral surfactants on enzyme activity is reported.[14] In our previous studies on the effect of cationic surfactants on α-CT-catalyzed hydrolysis of *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) we showed that not only the charge of the surfactant affects enzyme activity, but also the size of the head group has relevance.[15,16] In particular, among cationic cetyltrialkylammonium bromide surfactants, cetyltrimethylammonium bromide (CTABr) showed a deactivating effect on the enzyme. On the contrary, it was reported that the instantaneous activity was significantly favored in the presence of cetyltributylammonium bromide (CTBABr) both below and above the CMC. Moreover, the curve of reaction rate *versus* surfactant concentration was bell-shaped. At low surfactant concentrations, the presence of few CTBABr molecules can induce enzyme superactivity, while the reduction of reaction rate with increasing surfactant concentration was attributed to the partition of the substrate in the micellar aggregates. These results proved that an increase in the size of the trialkylammonium head group was responsible for α-CT superactivity.

The positive effect of a bulky head group was also observed for the hydrolysis of *p*-nitrophenyl acetate (PNPA) in solutions of ammonium and phosphonium surfactants.[17–19] The highest values of superactivities were observed with dodecyltriphenylphosphonium bromide and cetyltributylphosphonium bromide and such effects were due to an increase in the catalytic rate constant (k_{cat}).

Based on the previously reported positive influence observed for CTBABr on α-CT activity, the effect of tetrabutylammonium bromide (TBABr) was also studied.[20] This organic salt has the same head group of CTBABr but it is unable to form micelles because of the lack of the hydrophobic chain. Enzyme activity

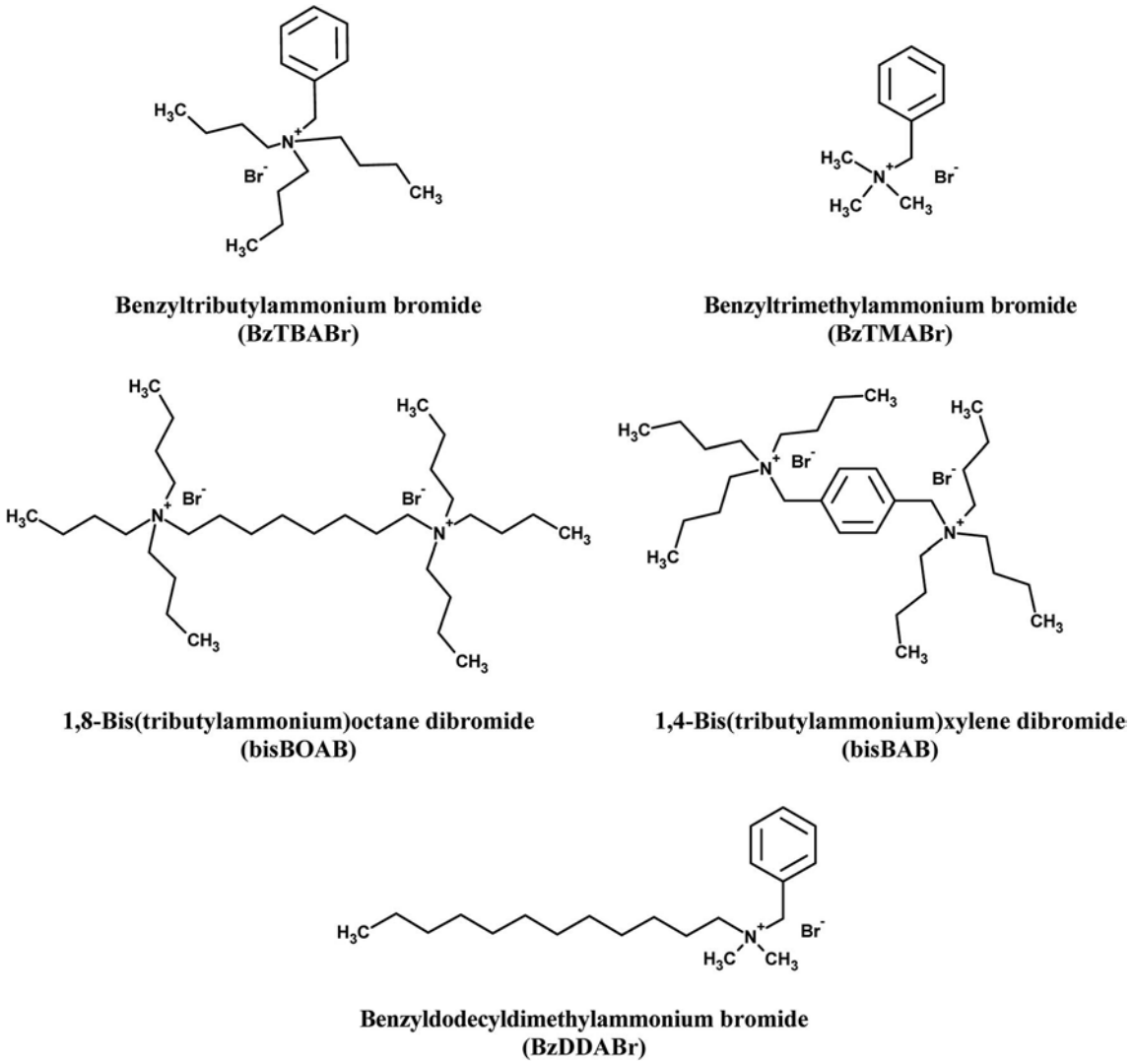

Fig. 1 Structures of the additives used in this work.

showed similar effects on hydrolysis rate to those obtained with CTBABr, confirming the activating role of a bulky, hydrophobic, ammonium group.

Different authors developed a different strategy to change the surfactant hydrophobic/hydrophilic balance, without altering the characteristics of the micellar interface, by modifying the hydrocarbon chain length.[21,22] Unfortunately, their results did not agree with each other. The hydrolytic activity of α-CT was studied in the presence of alkyldimethylethanolammonium bromide surfactants with different chain length (from C-10 to C-16) at concentration below and above the CMC. The k_{cat} increased with the surfactant chain length, confirming that a surfactant with higher hydrophobicity induces enzyme superactivity.[21] An increase in α-CT activity was also observed in presence of three alkyltrimethylammonium bromide surfactants (C_nTABr) with different chain lengths (n = 12, 14, 16). The maximum catalytic efficiency was registered at concentrations closer to surfactant CMC. However, in this case, the increase in surfactant chain length led to a lower superactivity.[22]

The characteristics of the substrate are also important in determining the effect of surfactants on α-CT hydrolysis rate.[23–25] In the presence of the same surfactant and at the same concentration (above the CMC), the distribution of the substrate between aqueous and micellar pseudophase strongly depends on its shape, charge and hydrophobicity. Therefore, the amount of free substrate available for catalysis, and then the kinetic behavior of the enzyme, could be very different.

Recently, Valiullina and co-workers carried out a study on α-CT catalyzed hydrolysis of *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA) in aqueous solutions of gemini surfactants, reporting that the length of both the alkyl chain and the polymethylene spacer affects the enzyme activity.[26,27] In particular, it was reported that the hydrolysis rate increased in the presence of surfactant with shorter alkyl chain (C-10 and C-12), while reaction was inhibited with additives with longer hydrocarbon radicals. These effects are closely related to surfactant concentration. Monomers in fact positively interacted with the enzyme improving its catalytic activity; on the other hand, the increase of micellized surfactant reduced the amount of free substrate, decreasing the hydrolysis rate. Moreover, α-CT activity was affected by the length of the polymethylene moiety between the two ammonium groups of hydroxyl-containing alkylammonium gemini surfactants. In this case, substrate hydrolysis was enhanced in the presence of additives with 10 and 12 methylene groups, while gemini surfactants with shorter spacers inhibited the enzyme. Substrate concentration in the aqueous phase decreased with increasing the length of the spacer and it was, again, the main factor leading to a reduction in the enzyme activity.

The present study aimed to gain a more extended insight into enzyme–cationic additive interactions. α-CT catalyzed hydrolysis of GPNA was studied in the presence of three additives bearing a benzyl moiety on the ammonium ion. Benzyltributylammonium bromide (BzTBABr), benzyltrimethylammonium bromide (BzTMABr) and the surfactant benzyldodecyldimethylammonium bromide (BzDDABr) were selected. Moving forward on the studies of specific synthetic molecules for specific roles, synthetic 1,8-bis(tributylammonium)octane dibromide (bisBOAB) and 1,4-bis(tributylammonium)xylene dibromide (bisBAB) were employed to study their effect on enzyme activity and stability. These molecules have two tributylammonium head groups, constrained by a spacer of different flexibility. Structures and acronyms of all the additives employed in this paper are reported in Fig. 1.

Finally, molecular modelling studies were performed to evaluate the most favorable binding poses of the additives in the enzyme catalytic site in the attempt to rationalize results derived from kinetic studies.

Experimental

Crystalline bovine pancreatic α-chymotrypsin (EC 3.4.21.1) (α-CT, 24.8 kDa, type II: 3 times crystallized, dialyzed and lyophilized) and the substrate *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) are purchased from Sigma. Buffered solutions of enzyme and substrate were freshly prepared immediately before use. The chemical Tris used for buffer preparation and *p*-nitroaniline used for molar absorption coefficient determinations are from Aldrich. The commercial grade salts benzyltributylammonium bromide (BzTBABr) and benzyltrimethylammonium bromide (BzTMABr) and dodecylbenzyldimethylammonium bromide (DDBzABr), are from Sigma-Aldrich and purified by crystallization; synthetic 1,8-bis(tributylammonium) octane dibromide (bisBOAB) and 1,4-bis(tributylammonium) xylene dibromide (bisBAB) have been prepared in our laboratories by direct quaternization from respective dibromides, *i.e.* 1,8-dibromooctane and α,α′-dibromo-*p*-xylene, with tributylamine in slight excess. Both the additives are purified and characterized.

1,8-Bis(tributylammonium)octane dibromide (bisBOAB): mp = 110–112 °C

^{1}H-NMR (CD_3OD, 200 MHz) δ = 0.92 (tr, 18H, $6CH_3$); 1.22–1.42 (m, 20H, $10CH_2$); 1.49–1.65 (m 16H, $8CH_2$); 3.11–3.23 (m, 16H, $8CH_2$).

1,4-Bis(tributylammonium)xylene dibromide (bisBAB): mp > 220 °C

^{1}H-NMR (CD_3OD, 200 MHz) δ = 0.92 (tr, 18H, $6CH_3$); 1.22–1.40 (m, 12H, $16CH_2$); 1.49–1.65 (m 12H, $6CH_2$); 3.50–3.60 (m, 12H, $6N–CH_2$); 5.20 (s, 4H, $2Ar–CH_2–N$); 7.80 (s, 4H, Ar).

α-Chymotrypsin activity assay

The α-CT activity measurements have been carried out spectrophotometrically at 25.0 ± 0.1 °C, following the increase in absorbance at 410 nm of the hydrolysis product of GPNA, *p*-nitroaniline (*p*NA). The molar absorption coefficient (ε_{410}) is 8800 M^{-1} cm^{-1} in pure buffer and any variations in presence of the additives at the concentrations used in kinetic measurements are evaluated and considered in rate values estimations. Measurements are performed with a Shimadzu UV-160A UV-VIS spectrophotometer equipped with a thermostated cell.

α-CT activity assay mixture was prepared in 0.1 M Tris–HCl buffer at pH 7.75; the enzyme concentration was always 0.2 mg

ml^{-1} (8 μM) and the concentration of the substrate GPNA was 2.5×10^{-3} M (unless otherwise specified). The reaction starts by enzyme addition from stock solution (10 mg ml^{-1}) to a pre-thermostated solution of substrate. The linear increase of absorbance at 410 nm due to *p*NA formation is then recorded as a function of time for 300 seconds; reaction rate of α-CT, defined as moles of *p*NA formed per unit of time, is calculated from the slope of the initial linear curve of *p*NA concentration *vs.* time.

Kinetic parameters k_{cat} and K_M in pure buffer and in presence of additives were obtained from the linear regression analysis of the double reciprocal Lineweaver–Burk plots in a range of substrate concentration between 0.1×10^{-3} M and 2.5×10^{-3} M.

All sets of experiments were reproduced at least three times and the differences between duplicates in each experiment were always below 5%.

α-Chymotrypsin stability assay

The 0.2 mg ml^{-1} enzyme solutions were prepared either in pure buffer or in the presence of the additives and incubated in a water bath at 25.0 °C. Equal aliquots were periodically withdrawn and injected to a thermostated substrate solution; the α-CT residual activity was measured as described in the former section.

Molecular modelling studies

The possible binding poses of the tested additives in the surrounding of the α-CT catalytic site were explored using the FLAP (Fingerprints for Ligands and Proteins) software, which is developed and licensed by Molecular Discovery Ltd. (http://www.moldiscovery.com). The docking procedure based on the FLAP algorithm[28] was first used to generate the GRID Molecular Interaction Fields (MIFs) for the α-CT cavity (pdb code: 4CHA, chains A, B and C). The MIFs inspection allows to visualize and quantify the hydrophobic and hydrophilic regions as well as the regions where H-bond donors and acceptors might occur. In this study, a FLAP pocket centered on the Ser-195 (erosion = 15, thickness = 5) was defined to select the region for MIFs calculation and docking runs. The probes used to generate the molecular interaction fields were H (shape), DRY (hydrophobic interactions), N1 (H-bond donor) and O (H-bond acceptor) interactions. Thus, α-CT cavity was used as a template, and the binding poses of the additives were generated using FLAP in the structure-based mode.[29–31] A maximum of 50 different conformations for each additive was generated and docked, to mimic flexibility. The obtained FLAP binding poses were ranked by the Glob-Prod descriptor, accounting for the best compromise between hydrophobic, hydrophilic, H-bond donor and H-bond acceptor interactions, and the binding pose with the highest similarity score was selected for each compound. In all cases, Glob-Prod values corresponding to the selected poses was greater than 0.9 (Glob-Prod scale from 0 to 1). In addition, the 10 top-ranked poses for each additive were visually inspected, to evaluate the variability in result. Variability in the orientation of the additives among the 10 top-ranked poses, when occurred, is discussed.

Results and discussions

Effect of cationic additives on α-chymotrypsin catalytic properties

As previously reported,[20] tetralkylammonium additives, with or without a hydrocarbon chain, increased enzyme activity when alkyl groups were rather bulky and hydrophobic. Inserting an aromatic ring on additive head group could lead to a further increase in hydrophobic/hydrophilic balance on additive structure, so we performed kinetic studies on aromatic-derived additives.

Benzyl-derived additives. The effect of BzTMABr, BzTBABr, BzDDABr on α-CT catalytic properties was investigated and results were compared with those previously obtained with TMABr, TBABr and CTABr, respectively.[15,20] BzDDABr, a surfactant with a C-12 alkyl chain, was selected instead of the C-16 derivative because of the very low solubility of the latter.

Fig. 2 shows profiles of α-CT activity as a function of additive concentration, in the presence of BzTBABr, BzTMABr and BzDDABr. In this plot, as in the following ones, the enzyme activity is quoted as the ratio between the reaction rate in the additive solution and in pure buffer.

In all cases, the introduction of a benzyl group into the additive structure produced a superactivating effect on the enzyme, represented by a bell-shaped curve as the additive concentration increases. Regarding BzTBABr, two different positive effects on enzyme activity were observed if compared with the "homologue" TBABr. First, a higher superactivation was attained since 12-fold increase in the reaction rate was registered, compared to the 8-fold one previously reported with TBABr (with respect to values in pure buffer). Moreover, the maximum of activation was reached at a lower concentration (0.15 M for BzTBABr *vs.* 0.4 M for TBABr). On the other hand, a complete change in activity trend was observed in the presence of BzTMABr, if compared with its tetramethyl homologous TMABr. TMABr produced only a slight and progressive increase

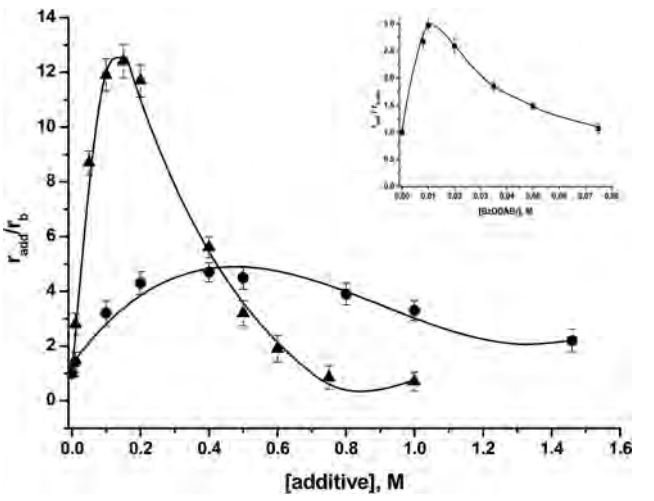

Fig. 2 Effect of BzTBABr (▲), BzTMABr (●) and, in the inset, BzDDABr (■) concentration on the activity of α-chymotrypsin in 0.1 M Tris–HCl buffer, pH 7.75 at 25.0 °C.

Table 1 α-Chymotrypsin kinetic parameters in the presence of additives and in pure buffer at 25.0 °C

Additive	Concentration, M	K_M, mM	10^2 k_{cat}, s^{-1}	$k_{cat(add)}/k_{cat(b)}$	k_{cat}/K_M, M^{-1} s^{-1}
—	—	0.44	1.46	—	33.2
BzTMABr	0.40 M	1.37	8.70	6.0	63.5
BzTBABr	0.15 M	1.99	31.10	21.3	156.3
BzDDABr	0.01 M	0.49	6.70	4.6	136.7

in α-CT activity, likely due to a simple salt effect,[20] while a 5-fold enzyme activation was gained with BzTMABr at a concentration of 0.4 M.

Similar positive outcomes were obtained also in the presence of a surfactant with an aromatic ring in the head group. BzDDAB, unlike CTABr, not only produced a 3-folds α-CT superactivation, but this effect was registered at a lower concentration with respect to tetralkylammonium salts, *i.e.* 0.01 M, probably because the presence of a long hydrocarbon chain could promote enzyme–additive association.

Kinetic parameters were also determined. Table 1 reports enzyme–substrate affinity (Michaelis constant, K_M), turnover number (k_{cat}), ratio between k_{cat} values in the presence of additive and in pure buffer ($k_{cat(add)}/k_{cat(b)}$) and k_{cat}/K_M values. Experiments were performed at the additive concentration that produced the maximum of superactivation.

In the presence of all investigated additives, enzyme–substrate affinity decreased but at the same time higher k_{cat} values than pure buffer were observed. In previous studies, similar changes in kinetic parameters are also reported for TBABr aqueous solutions.[20] To explain the positive effects reported, it was supposed that the closeness of the additive to the active site led to an increase in the hydrophobicity of the latter and so to a higher nucleophilicity of the hydroxyl group of Ser-195, mostly responsible for enzyme activity. On the other hand, the registered decrease in the affinity between enzyme and substrate could be explained either with direct interactions between additive and substrate or with some negative effect of the additive on enzyme conformation.

Experimental data reported in Fig. 2 show a 12-fold activation in the presence of BzTBABr, while, as evident in Table 1, the reaction rate ratio calculated from Lineweaver–Burk plot was significantly higher, *i.e.* about 20. Such disagreement is only apparent, since $k_{cat(add)}/k_{cat(b)}$ value is referred to saturation conditions of substrate, which means too high concentrations to be employed in the kinetic experiments. On the contrary, the calculated reaction rate ratios in the presence of BzTMABr were not notably different from the experimental ones, essentially since the increase in K_M value in this case was smaller. The reported superactivation with the benzyl-derived surfactant, BzDDABr, can be addressed to an increase in k_{cat}, since the enzyme–substrate affinity remained unchanged, probably because of the very low additive concentration at which the maximum of enzyme activation was gained. The same positive effect of BzDDABr has been reported in literature for the hydrolysis of *p*-nitrophenyl acetate,[17] where the obtained $k_{cat(add)}/k_{cat(b)}$ was about 4-fold higher with respect to the value in pure buffer, in a way similar to what we observed in our study for the hydrolysis of GPNA.

Enzyme kinetic parameters in the presence of BzTBABr have been compared to those previously quoted with TBABr.[20] The increase in k_{cat} obtained with both additives was the same, *i.e.* 0.31 s^{-1} (20-fold with respect to pure buffer), but the superactivation effect produced by BzTBABr was higher than TBABr, *i.e.* 12.4 and 8-fold respectively. Such evidence could be explained with the corresponding K_M values, being the enzyme–substrate affinity threefold lower in the presence of 0.4 M TBABr (K_M = 6.1 mM) with respect to the benzyl-derived additive. As already pointed out for DDBzABr, the observed effect could be simply related to the lower concentration of BzTBABr which produced the highest α-CT activation, considering that the benzyl-derived additive could establish π–π interactions with aromatic residues in enzyme catalytic site, promoting therefore the association between the enzyme and the additive itself.

Diammonium-derived additives. As already pointed out, hydrophobic/hydrophilic balance of the additive structure plays a key role in determining the superactivation effect on α-CT. Therefore, in view of a further improvement in the catalytic performances of the enzyme, two new diammonium salts have been properly designed and synthesized: 1,8-bis(tributylammonium)octane dibromide (bisBOAB) and 1,4-bis(tributylammonium)xylene dibromide (bisBAB). They both present two tributylammonium head groups constrained by a different spacer. BisBOAB possesses a saturated eight carbon atoms chain separating the two ammonium groups, while in bisBAB the same number of carbon atoms are arranged in a benzene ring and so the hydrophobicity of the whole structure turn out to be further increased. The profiles of α-CT activity in the presence of different concentrations of bisBOAB and bisBAB are reported in Fig. 3.

Both diammonium salts caused a marked increase in the rate of the hydrolysis reaction with a 9-fold and a 6-fold superactivation in the presence of bisBOAB and bisBAB respectively. In

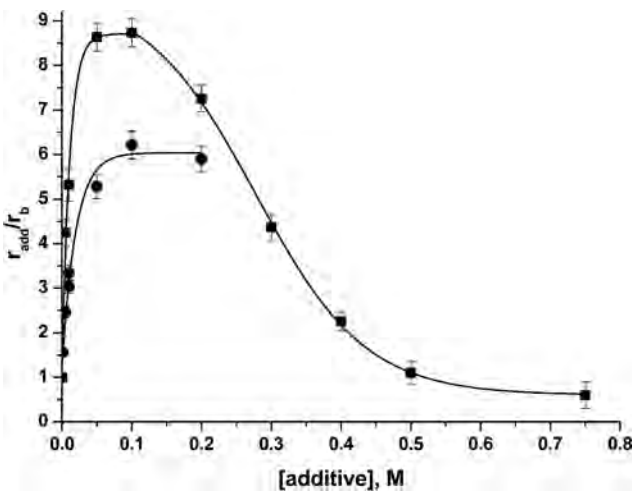

Fig. 3 Effect of bisBOAB (■) and bisBAB (●) concentration on the activity of α-chymotrypsin in 0.1 M Tris–HCl buffer, pH 7.75 at 25.0 °C.

particular, a bell-shaped trend of activity was obtained by varying bisBOAB concentration; the maximum superactivation value reached was the same of the one in the presence of TBABr. Nevertheless, as it could be expected, the concentration at which the maximum activity was achieved was four times lower, probably because of the synergic effect of two tributylammonium groups on the same single additive molecule.

As regards bisBAB, its poor solubility did not allow to obtain data at concentrations higher than 0.2 M; but in any case the rate of reaction achieved was lower than in the presence of bisBOAB.

Based on the above reported results with benzylic additives, it would be reasonably to expect a higher superactivating effect of bisBAB on enzyme with respect to bisBOAB. The obtained opposite result could be explained with the restricted conformational freedom degree of the two bulky, hydrophobic head groups separated by the aromatic ring, which could not allow them to fully display their effect on instantaneous enzyme activity.

Kinetic parameters are also measured and the obtained results are reported in Table 2.

As already described in the case of monoammonium salts, quite analogues effects on the enzyme catalytic properties were produced also by the new diammonium additives, *i.e.* an increase in the conversion rate of the substrate and, at the same time, a reduction in the enzyme–substrate affinity. Again, a noteworthy undervaluation in the superactivation effect, about 50–70%, was observed with respect to the experimental data reported in Fig. 3, and the same above accounted rationale could be provided to explain such evidence, that is the lack of saturation conditions of the substrate in the kinetic experiments.

Moreover, an interesting comparison could be stated between the obtained data in 0.4 M TBABr and in 0.1 M bisBOAB. In both cases, enzyme relative activity was about 9-fold higher than in pure buffer, but the presence of monoammonic additive TBABr in the solution would have led to an instantaneous activity of α-CT significantly higher than in bisBOAB, if saturation conditions could be achieved, since their $k_{cat(add)}/k_{cat(b)}$ ratios were 21 and 13, respectively.

As already pointed out, the degree of conformational freedom of the additive structure certainly plays a relevant role in the positive effect of the additive itself on enzyme activity. Therefore, notwithstanding the notable lower concentration at which the maximum of superactivation was reached, it can be hypothesized that the just described effect was dependent on the restricted mobility of the two tributylammonium head groups in bisBOAB, if compared with the single, totally free tetrabutylammonium ions.

Table 2 α-Chymotrypsin kinetic parameters in the presence of additives and in pure buffer at 25.0 °C

Additive	Concentration, M	K_M, mM	$10^2\ k_{cat}$, s^{-1}	$k_{cat(add)}/k_{cat(buffer)}$	k_{cat}/K_M, $M^{-1}\ s^{-1}$
—	—	0.44	1.46	—	33.2
BisBAB	0.1 M	1.49	12.0	8.2	80.5
BisBOAB	0.1 M	1.86	19.3	13.2	103.8

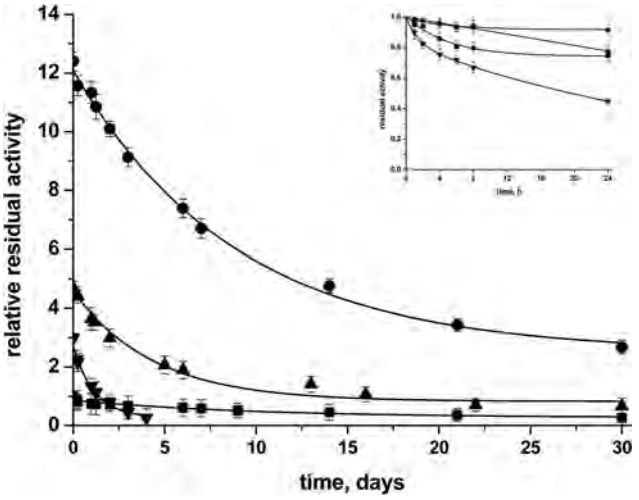

Fig. 4 Residual activity of α-chymotrypsin in 0.15 M BzTBABr (●), 0.4 M BzTMABr (▲), 0.01 M DDBzABr (▼) and pure buffer (■) as a function of storage time at 25.0 °C. In the inset, the enzyme residual activity in the first 24 h is also reported.

Enzyme stability

Enzyme stabilization is one of the most important topics in basic and applied enzymology and understanding any relationship between the structure of the additive and its effect on enzyme stability is of particular relevance.

Benzyl-derived additives. Fig. 4 depicts the time course of α-CT activity in pure buffer and in aqueous solutions containing BzTBABr, BzTMABr and DDBzABr as well. The ratio between the instantaneous specific reaction rate and the one in pure buffer at zero time is reported as function of storage time in order to easily compare the deactivation kinetics in the different media and to display superactivity preservation during storage time. In the inset, the ratio of instantaneous to initial rate in the first 24 h is also reported. Stability was monitored at 25.0 °C and the investigated additive concentrations were those reported in the caption of the figure.

In the absence of additives and in the first 24 h, α-CT lost 25% of its initial activity; then, activity continued to decrease, until, after 30 days, the enzyme retained less than 30% of its activity.

Enzyme deactivation in the presence of the surfactant DDBzABr was even faster, since relative residual activity decreased of about 30% in the first 8 h, and, after 96 h, it was even negligible. On the contrary, the benzyl-derived salts allowed the enzyme to retain almost all of its activity during the first hours of incubation, and the superactivation levels observed in the kinetic experiments were maintained. After one month of incubation, the percentage of residual enzymatic activity was lower in the presence of BzTBABr and BzTMABr than in pure buffer, between 15 and 20%. Therefore, although the presence of a benzyl group as substituent on additive head group at first produced an increase in instantaneous activity, in the long period it speeded up the α-CT deactivation. Nevertheless, at least in BzTBABr solutions, after 30 days residual activity was about 3 times higher than that in pure buffer at time zero, which means that the enzyme was still superactivated.

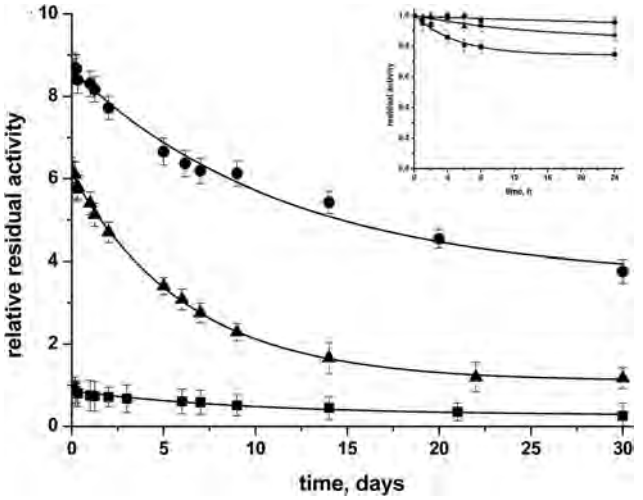

Fig. 5 Residual activity of α-chymotrypsin in 0.1 M bisBOAB (●), 0.1 M bisBAB (▲) and pure buffer (■) as a function of storage time at 25.0 °C. In the inset, the enzyme residual activity in the first 24 h is also reported.

Diammonium-derived additives. Finally, α-CT was incubated in the presence of 0.1 M bisBOAB and 0.1 M bisBAB and the enzyme residual activity was monitored as a function of time. The corresponding profiles are showed in Fig. 5 and data in pure buffer are also reported for comparison. As in Fig. 4, the inset shows the ratio of instantaneous to initial specific reaction rate in the first 24 h.

In the presence of both the additives, only a slight loss of enzymatic activity was observed in the first 48 hours, 10 and 20% for bisBOAB and bisBAB respectively, compared with 30% decrease in pure buffer. Interestingly, in the long period, trends of residual activity in the presence of bisBOAB and the homologue monoammonic TBABr are quite comparable,[20] since in the presence of bisBOAB enzyme retained almost 45% of its initial instantaneous activity even after one month of incubation.

As regards bisBAB, after the first 48 h the deactivation rate was markedly higher than those in pure buffer and in bisBOAB and, after 30 days, the residual activity was only about 16% of the initial value. However, although the superactivation effect was almost completely lost in bisBAB, after one month the enzyme activity still remained higher than the starting value in pure buffer.

The obtained results with the two di-tributylammonium salts suggested that, even though the degree of flexibility of the spacer did not strongly affect enzyme superactivation, it seems to be of primary importance in determining its deactivation rate. Moreover, the aromatic ring itself could negatively affect α-CT stability, as suggested by the corresponding experiments with the monoammonic benzyl-derived additives (see Fig. 4).

Molecular modelling studies

Experimental data collected suggested that the enzyme superactivation effect could be related to a possible variation of the catalytic cavity environment upon interaction with certain additives. In particular, it was hypothesized that additives could interact with the enzyme making the region close to the catalytic triad more hydrophobic, and thus favoring the catalytic activity.

Therefore, we attempted to rationalize this hypothesis *in silico*, studying the possible binding modes of the tested additives in the catalytic site environment. Based on the experimental conditions used for our tests, in which the additives were added to the enzyme prior to substrate addition, we decided to model the interaction between each additive and the enzyme in the absence of the substrate. The X-ray structure of un-complexed α-CT (PDB code: 4CHA) was selected because of its high quality resolution (1.68 Å) and because already used as a valuable structure for modelling studies.[32–35] The FLAP (Fingerprints for Ligands and Proteins) algorithm has been extensively described elsewhere,[28] and was used first to identify the cavity surrounding the catalytic triad, and then to evaluate the most favorable binding poses of the studied additive in the selected region.

Briefly, FLAP is a virtual-screening and model-development method based on 3D molecular similarity, measured through common Molecular Interaction Field (MIF) volumes.[28–31] In a structure-based approach, MIFs representing the regions of protein cavity (here α-CT) suitable for hydrophobic, hydrophilic, H-bond donor or H-bond acceptor interactions are compared with analogue MIFs generated on the ligand structure (here the additive molecule); thus, the similarity between MIFs of the protein and MIFs of the ligand is expressed in terms of a similarity score (here the Glob-Prod score). Since several binding poses might be produced by FLAP for a ligand, in this study for each ligand the correspondent FLAP results were ranked by the Glob-Prod similarity score, and the pose with the highest similarity score was extracted.

Benzyl-derived additives. Fig. 6A reports the hydrophobic regions in terms of MIF (green volumes) close to the catalytic moiety of the enzyme in the un-complexed state, while the FLAP binding poses for benzyl-derived additives are shown in Fig. 6B–D.

When hydrophobic MIFs are generated in FLAP, it is possible to define which amino acids contribute to a local hydrophobic effect. Concerning the α-CT cavity, in addition to a minor hydrophobic region generated by the aromatic ring of H57, the main hydrophobic region is generated by W215, directly facing the catalytic region. A further additional elongated hydrophobic region was observed closely (Fig. 6A). The modelling studies for the benzyl-derived additives showed that BzTBABr, BzTMABr and BzDDABr favorably orient their aromatic moiety towards W215, leading to an efficient π–π stacking. The orientation of the benzyl-derived additives is then influenced by the other surrounding hydrophobic regions, depending on the nature of the additive. For example, for BzTMABr several binding poses with very similar FLAP similarity score (and thus to be considered almost equivalent) were obtained, preserving the interaction with W215 but having the trimethylammonium group variably oriented (data not shown). Fig. 6B illustrates the top-ranked pose for BzTMABr highlighting that the trimethylammonium group, due to its small size and its low hydrophobicity, cannot favorably interact with other hydrophobic regions. On the contrary, the best binding pose (highest

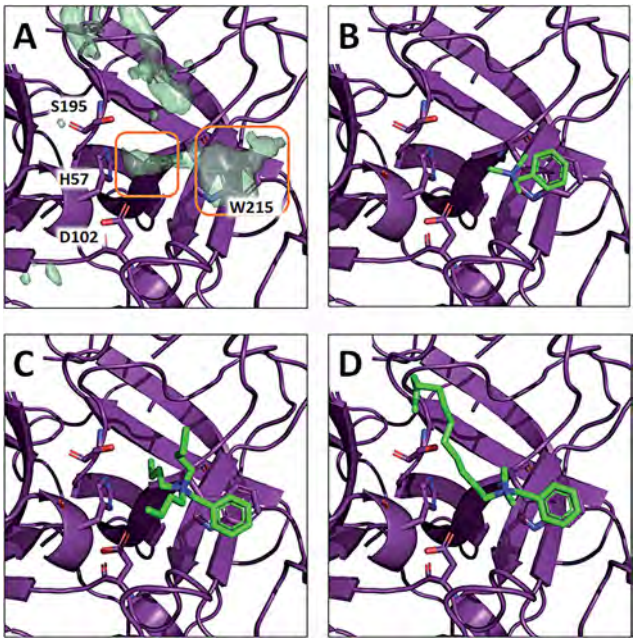

Fig. 6 Modelling of the α-CT/additive interaction using FLAP. (A) Representation of the hydrophobic MIF (green volumes) close to the α-CT catalytic site. (B–D) FLAP binding poses for BzTMABr, BzTBABr and BzDDABr, respectively.

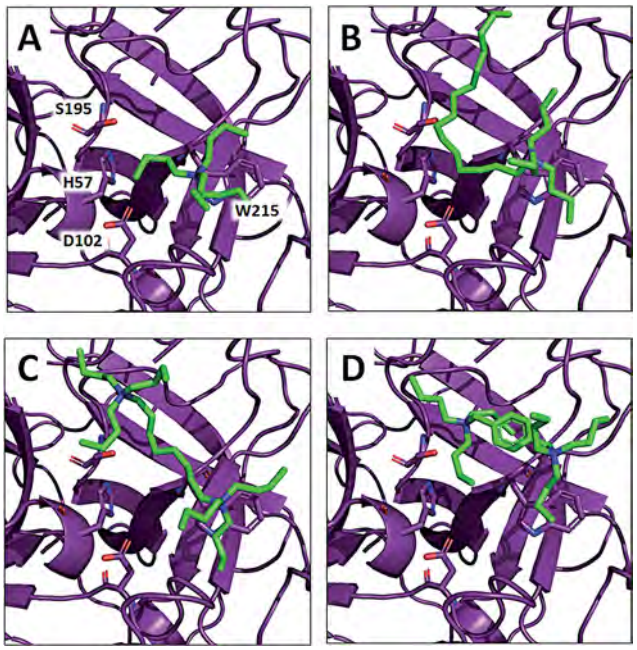

Fig. 7 FLAP binding poses for TBABr (A), CTBABr (B), bisBOAB (C) and bisBAB (D).

similarity score) for BzTBABr is reported in Fig. 6C, with the shown orientation being preserved among the 10 top-ranked FLAP poses. In this case, the tributylammonium moiety perfectly points towards the hydrophobic region generated by H57, due to its more hydrophobic nature and to the rigid structure of this additive. Finally, BzDDABr displayed a similar binding pose with respect to BzTBABr (Fig. 6D); however, the high flexibility of the alkyl chain allows the latter to be oriented either towards the H57 hydrophobic MIF (Fig. 6D) or towards the elongated one far from the catalytic triad (data now shown).

A comparison of the FLAP binding poses for the three benzyl-derived additives suggests that all the benzyl-derived additives increase the hydrophobicity of the active site, but at different level. In particular, BzTBABr is the compound able to most efficiently increase the overall hydrophobicity of the α-CT catalytic site. Indeed, it possesses three chemical features that force the compound to be oriented towards the catalytic site: (1) the aromatic portion to maximize the interaction with W215; (2) the tributylammonium group at a distance from the aromatic moiety that perfectly matches the hydrophobic region by H57; (3) a rigid nature that stabilizes this hypothetical binding mode. As a main result, the orientation of the tributylammonium group towards the catalytic triad might increase the overall hydrophobicity of that region, without hampering the substrate binding.

This analysis of the *in silico* models in Fig. 6 nicely correlates with the experimental kinetic parameters. In fact, as reported in Table 1, k_{cat} in the presence of BzTMABr and BzDDABr is about 5–6-fold higher than that in pure buffer, while in BzTBABr solutions the more hydrophobic environment could be responsible for the increase of k_{cat} of about 20-fold.

Diammonium-derived additives. Modelling studies were extended to the two novel dicationic salts, bisBOAB and bisBAB, and also to TBABr and CTBABr for comparative purpose. The top-ranked binding poses by Glob-Prod similarity score for the four compounds are reported in Fig. 7.

According to our model, notwithstanding the lack of an aromatic moiety, the tributylammonium groups of the additives are positioned in the hydrophobic MIF of W215. Of course, the extent of the additive/W215 chemical interaction results to be weakened. However, this W215 anchor point still allows the additive to be located close to the hydrophobic region of H57, producing an increase in the hydrophobicity of the active site and, consequently, enzyme superactivation. In case of small-size hydrophobic additives, such as TBABr and BzTBABr, their rigidity force them to be similarly located close to the catalytic triad region, and accordingly their k_{cat} are very similar (k_{cat} = 31.0×10^{-2} s^{-1} and 31.1×10^{-2} s^{-1}, respectively).

Concerning bisBOAB and TBABr, as previously reported the enzyme superactivation in solutions upon their addition was very similar, but the concentration at which the maximum is achieved in the presence of the dicationic salt was four times lower than TBABr. Modelling studies point out two effects that could explain the experimental behavior. Firstly, the symmetric nature of bisBOAB doubles the chances to get a favorable binding with the enzyme. In addition, the more hydrophobic bisBOAB can strongly interact with the hydrophobic regions in the protein cavity, stabilizing the orientation tributylammonium group toward W215. Consequently, a lower number of additive molecules is needed to produce the same superactivating effect. However, the hydrophobic small-size features of TBABr seemed to more efficiently increase the hydrophobicity of the catalytic site with consequent increase of the k_{cat} (31.0×10^{-2} s^{-1} for

TBABr and 19.3×10^{-2} s^{-1} for bisBOAB). A plausible hypothesis, as previously discussed for BzDDABr, is that the high flexibility of BisBOAB can be responsible for the lower hydrophobic effect at the catalytic triad region. Indeed, the long and flexible spacer between the two tributylammonium moieties can interact with either the hydrophobic region generated by H57 or the more extended one diagonally located (Fig. 6A), and according to the FLAP poses the second one seems to be more favorable (the pose with highest similarity score is diagonally oriented). Although in a dynamic context the spacer could switch between the two locations, the overall effect is a lower hydrophobic contribution on the catalytic triad.

In agreement with this hypothesis, CTBABr displayed a k_{cat} value very similar to that of bisBOAB and ($k_{cat} = 19.5 \times 10^{-2}$ s^{-1} and 19.3×10^{-2} s^{-1}, respectively). Indeed, as for BzDDABr, the high flexibility of the alkyl chains of CTBABr and bisBOAB actually allows a number of similarly ranked binding poses, with the aliphatic chains being oriented towards the H57 hydrophobic MIF or towards the elongated one far from the catalytic triad. Thus, Fig. 7B and C are representative of binding mode with the highest similarity score. However, the visual inspection of the ten top-ranked poses for these compounds proved that in all of them a tributylammonium moiety is located in correspondence of W215. Thus, an extensive flexibility of the additives generated by long alkyl chains seems to induce an unfavorable effect on the k_{cat}, both in the benzyl-derived and in the diammonium-derived series.

Although flexibility appears to be detrimental for k_{cat}, the more rigid bisBAB also displayed a low effect, with a k_{cat} value of 12.0×10^{-2} s^{-1}. Fig. 7D illustrates the FLAP binding pose for this compound. It is noteworthy that bisBAB, although a more rigid compound, displays the worst interaction with W215, probably due to its large size that make its direct exposition towards the catalytic site more difficult.

Conclusions

The positive effect on α-CT catalytic properties of cationic additives with a bulky hydrophobic group was here confirmed. The structure of quaternary ammonium-based additives has been properly varied by substituting an alkyl group with a benzylic one. The presence of an aromatic ring in the additive structure highly increased GPNA hydrolysis instantaneous rate. On the other hand, stability studies showed that it could cause a loss of enzyme catalytic performance in the long time probably due to structural modifications in α-CT. New synthesized diammonic additives (bisBOAB and bisBAB) produced enzyme superactivation at lower concentrations than monoammonic corresponding salts, although the introduction of a rigid spacer, as in the case of bisBAB, reduced such increase in instantaneous reaction rate. Moreover, in the presence of bisBOAB, enzyme residual activity was comparable with that in the presence of monoammonic tetralkylammonium salts even after one month, while an almost complete loss of superactivation effect in time was observed in the presence of bisBAB, likely due to the benzylic group, in agreement with results obtained with benzylic monoammonic additives.

Results suggested that enzyme catalytic properties could be strongly affected not only by the charge, the size and the hydrophobic/hydrophilic balance of additive head group, but also by specific interactions between enzyme and definite groups within the additive structure.

Molecular modelling studies performed suggest that W215 could be a key residue to anchor additives close to the catalytic site. According to the *in silico* evaluations, the α-CT superactivation by small size additives with reduced flexibility could be explained by the optimal exposition of the tributyl group towards the catalytic site, once the molecule is anchored to W215. This binding mode seems to be responsible for an increased hydrophobicity of the catalytic site, resulting in an increased k_{cat} value. Contrarily, more flexible additives and large rigid ones displayed a lower superactivation effect due to alternative binding modes for the alkyl chains.

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