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ORIGINAL ARTICLE

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Abstract The effect of aluminum ions on the binding properties of α-chymotrypsin has been studied. The results show that aluminum does not affect the catalytic rate constant k_{cat}, but it acts as an enzyme activator favoring the binding of the substrate to the catalytic site (i.e. decreasing K_m). Furthermore, aluminum binding to α-chymotrypsin displays about a threefold decrease in its affinity for the macromolecular inhibitor bovine pancreatic trypsin inhibitor (BPTI). Altogether, the different effect of aluminum on the binding of synthetic substrates (e.g. *N*-α-benzoyl-L-tyrosine ethyl ester, BTEE) and macromolecular inhibitors (e.g. BPTI) to α-chymotrypsin suggests the occurrence of an aluminum-linked conformational change in the enzyme molecule which brings about a marked structural change at the primary and secondary recognition sites for substrates and inhibitors. The modulative effect exerted by aluminum on the enzyme hydrolytic activity has been investigated also as a function of pH. The ion-linked effect appears to be dependent on the pH in a complex fashion, which suggests that aluminum binding is controlled by the protonation of at least two classes of residues on the enzyme molecule.

Key words Proteinases · Aluminum · Allosteric modulation · Metal pollution · Alzheimer's disease

Introduction

Aluminum ions have been proposed to be a factor of biological toxicity, contributing to the etiology of a variety of neurological and skeletal disorders, even though the molecular mechanisms underlying these processes are still unclear [1]. Aluminum can be found in living cells or biological fluids, both in free (mononuclear ions) or complexed form (low- or high-molecular-weight complexes) [2]. In aqueous media, in fact, aluminum can be found in several soluble ionic species, depending on the pH, and shows a preferential interaction with oxygen donor ligands. In biological systems, this requirement is fulfilled by carboxylate and phosphate groups which are responsible for the formation of physiologically meaningful complexes such as those formed with polyphosphates, including nucleoside triphosphates and citrate [2]. The aluminum transport occurs through the plasma, where the ion can bind the low-molecular-weight molecule citrate or the macromolecular protein transferrin, which firmly binds aluminum at sites unoccupied by iron [3] and appears to be responsible for the accumulation of the ion in cells and tissues [4]. Recently, it has been reported that aluminum could be an etiologic agent for Alzheimer's disease since (1) an association between the bioavailability of the metal and the incidence of the disease has been observed [5], and (2) the evidence of increased levels of the ion in the characteristic proteinaceous plaques, which are present in the brain of Alzheimer subjects, has been reported [6]. Using α-chymotrypsin as a model, it has also been suggested that the ion could affect the proteolytic processes apparently involved in plaque formation, either through a direct effect on the activity of the proteolytic enzymes [7] or by affecting the proteinase(s)/inhibitor(s) interaction [7]. On the other hand, the modulative effect exerted by metallic ions on proteolytic systems has been recently emphasized by many authors. It has been reported that several mono- and divalent cations, particularly sodium and calcium, bind proteolytic enzymes, inducing conformational changes which affect the catalytic activity of proteinases and/or the recognition process by macromolecular inhibitors [8–10]. Owing to our interest in the characterization of the effect of metal ions on the binding properties of α-chymotrypsin and in order to better understand, from a quantitative standpoint, the effect of alu-

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minum on proteolytic processes, in this paper we present a study of the effect of the metal on the binding properties of the proteinase α-chymotrypsin. This study, performed over a fairly wide pH range and analyzed through a linkage approach [11], indicates that aluminum binding to the enzyme molecule strongly affects only the recognition of the substrate, thus altering the overall catalytic properties of the enzyme and acting as an allosteric effector. In particular, it produces a positive allosteric effect on the binding of small synthetic substrates, whereas a decreased affinity is observed for the interaction with large macromolecular inhibitors. Furthermore, the heterotropic effect exerted by the ion appears to be dependent on pH, suggesting that at least two classes of protonating residues modulate the aluminum-linked effect. These data indicate, for the first time, that a complex functional modulation mechanism can be exerted on α-chymotrypsin by enviromental conditions, thus opening up an additional field of investigation into the effect of pollution on the widespread physiological functions of serine proteinases, which range from blood coagulation to enzymatic processing of neuropeptides and growth factors, and may be at the origin of several pathological disorders in man.

Materials and methods

Materials

Bovine α-chymotrypsin grade I and the synthetic substrate *N*-α-benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma (USA). The BPTI was a generous gift of Bayer (Wuppertal, Germany). The Chelex 100 resin was from Bio Rad (USA), and the HCl (Baker Instra Analyzed) used for ions depletion was from Baker (USA). Terbium chloride and aluminum chloride were obtained from Aldrich (USA). All buffer solutions were prepared with deionized water which was further distilled in an Aquatron distiller (Bibby Sterilin, England). All other chemicals were of analytical grade.

Methods

Preparation of stripped α-chymotrypsin

The commercial enzyme was freed of ions by dissolving the lyophilized protein in 1 mM HCl (Baker Instra Analyzed) and extensively dialyzing it for 5 h against the same solution containing 4 g/l of Chelex 100 resin. The stripped protein was maintained at −30 °C and used as soon as possible.

Determination of the enzyme activity

The chymotryptic activity was assayed, as previously reported, following (at 256 nm and 25 °C) the hydrolysis of the synthetic substrate BTEE [12]. When aluminum was added to the assay, 5 min preincubation of the enzyme with aluminum was performed in order to reach the equilibrium. When the effect of metal on the BPTI-α-chymotrypsin interaction was studied, a solution of the enzyme in 50 mM 3-(*N*-morpholine) propanesulphonic acid (Mops)-NaOH buffer (pH 6.5), was incubated for 5 min with increasing amounts of the ion. A solution of BPTI was then added, and, after an additional 5 min, the residual activity of α-chymotrypsin was measured as reported above. The values of the equilibrium dissociation constants of the α-chymotrypsin-BPTI complexes were determined by measuring the inhibitory effect of BPTI on the enzymatic hydrolysis of BTEE according to Bieth [13]. The concentrations of α-chymotrypsin and BPTI were determined spectrophotometrically using an extinction coefficient at 280 m ($E^{\%}_{cm}$) of 20.4 for the enzyme and of 8.3 for the inhibitor, respectively. Aluminum was added as the chloride.

Fluorimetric determinations

Fluorimetric experiments were performed on an ISS-Greg 200 spectrofluorimeter at 25 °C. Titrations of the free enzyme were performed by adding small aliquots (2–5 μl) of a concentrated solution of $AlCl_3$ to 2 ml of the solution of the enzyme. After each addition, the luminescence intensity was recorded for a period of 4 min, checking for the absence of protein precipitation. All the experiments were performed at 25 °C (±0.1 °C) in 50 mM Mops/NaOH buffer, pH 6.5. Emission spectra were obtained using an excitation wavelength of 290 nm. The binding of terbium to α-chymotrypsin was observed following the enhancement of the lanthanide luminescence due to energy transfer from the protein to the cation and recording the emission spectra in the wavelength range 530–560 nm. ($\lambda_{Exc}=285$ nm). The displacement of aluminum from the enzyme by the lanthanide was followed, recording the decrease of the luminescence at 547 nm due to the addition, in small aliquots, of an aqueous solution of terbium chloride to a solution of the enzyme saturated with aluminum. The values of the binding constants of aluminum ions to the enzyme were calculated according to De Jersey et al. [14].

Results and discussion

Aluminum binding to α-chymotrypsin has been explored either through fluorimetric measurements or studying the effect of the ion on the hydrolytic activity of the enzyme towards the synthetic substrate BTEE. The fluorimetric experiments were performed directly titrating the free enzyme with a concentrated solution of aluminum chloride. Aluminum binding probably induces a molecular rearrangement of the enzyme molecule which involves also tryptophan residues, as can be seen in Fig. 1A, where the emission spectra of the enzyme in the presence of increasing concentrations of aluminum are reported. The association binding constant of the ion to the enzyme calculated from these experiments is $3.6\pm0.6\times10^4\ M^{-1}$ (see Fig. 1B and Table 1).

In order to elucidate the nature of the binding site, titrations with terbium of the free enzyme in the absence and in the presence of increasing amounts of aluminum have been performed. Thus, it has been reported that terbium, like all other lanthanides, binds α-chymotrypsin on a specific site, which appears to be the same as that bound with calcium [10]. The results obtained, summarized in Table 1, indicate that the binding of aluminum does not hinder the binding of terbium, and that the lanthanide luminescence is enhanced by the presence of bound aluminum. These data suggest the presence in the enzyme of two distinct but interacting sites where the two cations are able to bind. On the other hand, the presence of a common site for aluminum and terbium appears unlikely also on the basis of

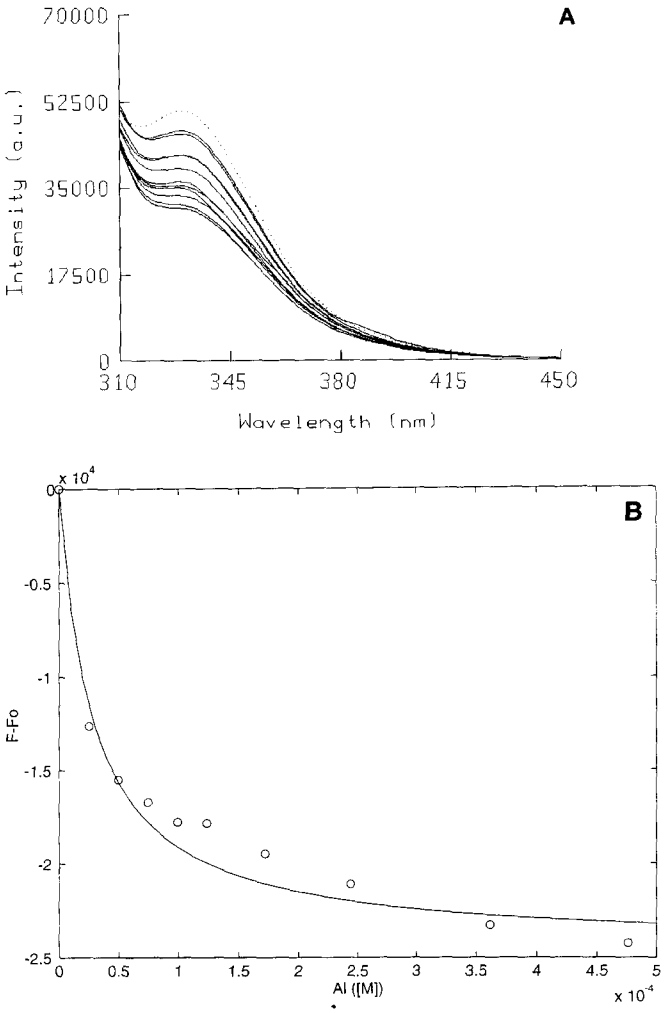


Fig. 1 **A** Effect of increasing amounts of aluminum on the emission spectra of α-chymotrypsin (---). **B** Luminescence changes due to aluminum binding. Titrations were performed as reported in Materials and methods using 10 μM enzyme

Table 1 Values of the equilibrium binding constants of aluminum and terbium to α-chymotrypsin as determined by fluorescence experiments. Experiments were performed at 293 K in 50 mM Mops-NaOH buffer, pH 6.5

	$K_L \times 10^{-4}$ (M^{-1})	$*\sigma K_L \times 10^{-4}$ (M^{-1})
Terbium	1.5	0.18
Aluminum	2.5	0.4
$K_{terbium}/K_{aluminum}$[a]	0.259	0.03

* σ represents the standard deviation for the parameters (68%), as reported in the text

[a] The value reported was obtained by the fluorimetric data following the displacement of aluminum by terbium

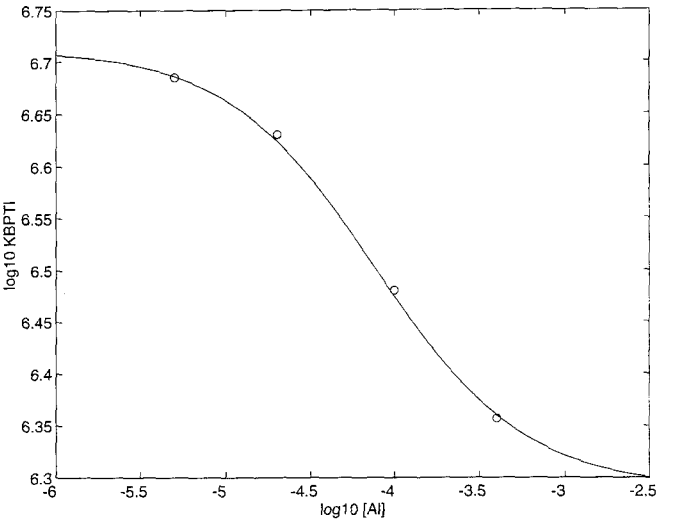


Fig. 2 Effect of increasing concentrations of aluminum on the equilibrium constant for BPTI binding to α-chymotrypsin. The experiments were performed, at 25 °C, in 50 mM Mops-NaOH at pH 6.5. The solid line originates from a non-linear best-fit of the experimental data performed according to Eq. 1

the smaller ionic size of aluminum (0.54 Å) than that of terbium (0.92 Å).

Like terbium and calcium [10], aluminum binding brings about a decrease in the BPTI binding affinity to α-chymotrypsin (Fig. 2), which can be analyzed according to the following linkage equation:

$$K_{obs} = K_0 \frac{1 + K_b[Al]}{1 + K_f[Al]} \quad (1)$$

where K_{obs} is the observed BPTI binding affinity, K_0 is the BPTI association equilibrium constant in the absence of aluminum, and K_b and K_f are the equilibrium constants of aluminum binding to BPTI-bound and BPTI-free α-chymotrypsin, respectively. This analysis indicates that the value of K_f ($=2.1 \times 10^4\ M^{-1}$) is two to three times that of K_b ($=0.8 \times 10^4\ M^{-1}$) and that aluminum acts as a negative allosteric effector on BPTI binding. On the other hand, the steric rearrangement caused by aluminum binding also produces an enhancement of the hydrolytic activity of α-chymotrypsin. This effect, also reported by Clauberg and Joshi [7], and ascribed to the effect of aluminum hydroxylated polynuclear species, appears to be dependent on the concentration of the ion.

In order to better characterize the effect of the aluminum molecular species involved in the α-chymotrypsin catalytic activity and to have a more descriptive picture of the parameters affecting all the equilibria involved, we have studied the pH dependence both of the binding and catalytic events. The treatment of the pH effect was based on an extension of the equilibrium theory of ligand binding and linkage [11] to macromolecular systems working at steady state, assuming that the reaction involving protons/ions dissociation-addition is very fast with respect to the catalytic events, as is very often the case with small ligands [15]. The linkage approach allows a quantitative description of the effect of linked ligands on the binding and catalytic properties of the enzyme at the reactive site, and parallels the description of the proton effect on the stability of various

proteinase-proteinase inhibitor complexes already studied in detail [17, 18]. The proton-linked effect of aluminum binding to α-chymotrypsin can be cast into a phenomenological scheme as reported in Scheme 1, where E^0 is the free enzyme, E^1 is the aluminum-bound substrate-free enzyme, E^0S is the aluminum-free substrate-bound enzyme, and E^1S is the aluminum-bound substrate-bound enzyme. All four species (i.e. E^0, E^1, E^0S, and E^1S) are characterized by one or more protonation constants (K_{Hi}), which are reported in the scheme without being defined in detail. Although the recognition mechanisms betweeen serine proteinases and substrates has been reported to be more complex than that reported in Scheme 1 [17], our analysis considers only one enzyme substrate-complex. In fact, under our conditions, the deacylation step is always faster than the acylation step, so that $k_{cat}=k_p$ and $K_S=1/K_m$ and the catalytic behavior of α-chymotrypsin can be accounted for by a Michaelis-Menten mechanism, with all the intermediates in the E and ES manifold in a quasi-equilibrium regime [16, 19]. The proposed scheme reports aluminum as Al^{3+} ion even though aluminum is involved in several pH-dependent equilibria, and the analytical problem is further complicated by a very low solubility constant ($K_{sol}=2\times10^{-32}$ M^4, at 298°K) for the aluminum hydroxide $Al(OH)_3$.

At steady state, the effect of ions on the catalytic activity of the enzyme hinges on the perturbation of the value of the apparent Michaelis-Menten constant ($K_{m,app}$) or, under particular experimental conditions, of the dissociation equilibrium constant of the enzyme-inhibitor complex (K_S), and of the apparent V_{max} value. The velocity equation can be derived either from a classical approach [20] or from a linkage analysis at steady state [15].

$$V=\frac{V_{max}\dfrac{f_{EP}}{f_{ES}}[S]}{\dfrac{1}{K_S}\dfrac{f_E}{f_{ES}}+[S]} \tag{2}$$

The analytical forms of $K_{S,app}$ and $V_{max,app}$ can be easily derived in the form shown below:

$$K_{S,app}=K_S\frac{f_{ES}}{f_E}$$

$$V_{max,app}=V_{max}\frac{f_{EP}}{f_{ES}}$$

where f_E, f_{ES} and f_{EP}, analogously to the thermodynamic description at equilibrium [20], are the subpolynomials of the manifold referring to the protonation equilibria taking place on the enzyme forms, upon the enzyme-substrate complex formation, and during the catalytic step, respectively. The functional expressions for the subpolynomials are easily derived as:

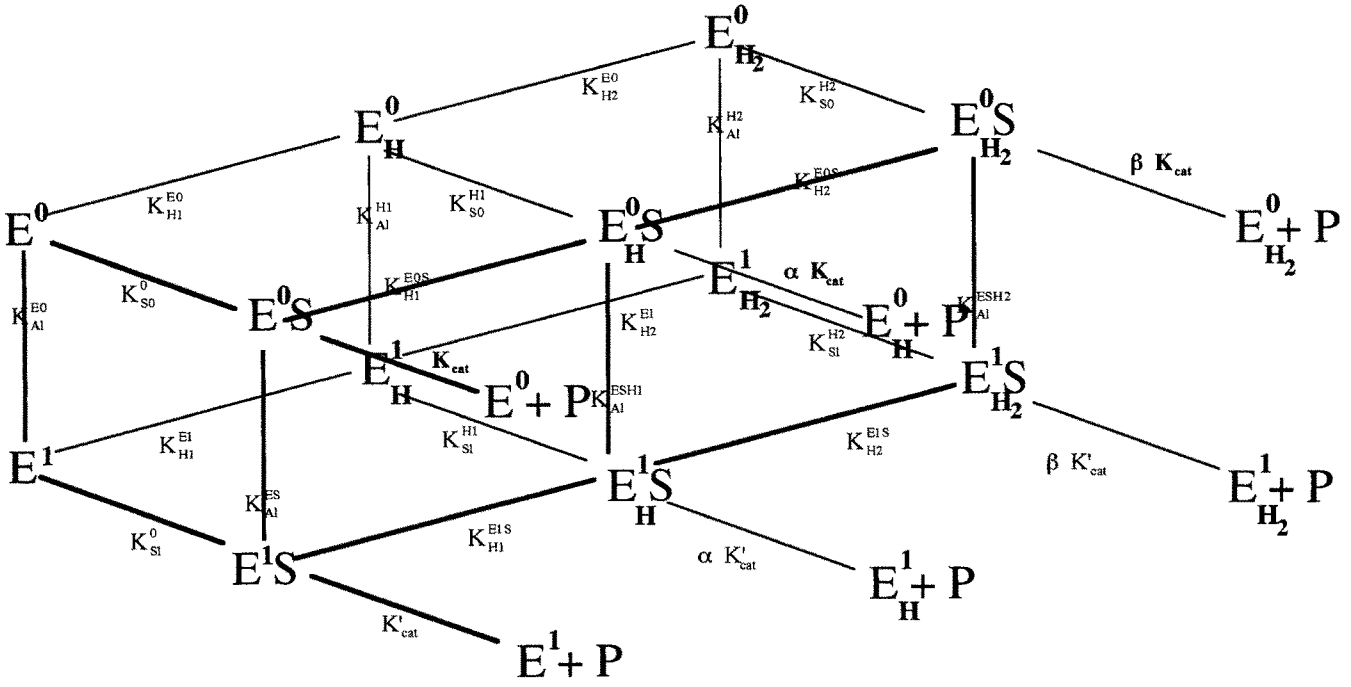
$$f_E=1+K_{Al}^{E0}[Al]$$

$$f_{ES}=1+K_{Al}^{ES}[Al]$$

$$f_{EP}=1+\alpha' K_{Al}^{ES}[Al]$$

The velocity equation (Eq. 2) can be used as a regression function of the kinetic data obtained at different

Scheme 1 Equilibria involved in the interaction between aluminum and α-chymotrypsin either in free- or substrate-bound form. The bold lines refer to the minimal scheme that can be depicted for the ligand- and proton-linked equilibria on the basis of the analysis of the experimental data (see text). For the significance of the species and of the equilibrium constants see text



ion chemical potential. This approach also allows a global analysis of all the experimental data obtained by a non-linear multiresponse data fitting [21], providing a strong constraint in the simultaneous fit and better parameter resolution [21]. In the case under study, steady-state velocity determinations were collected as a function of substrate concentration over the aluminum concentration range used. Typically, ten substrate concentrations (ranging between 2×10^{-5} M and 30×10^{-5} M) at a given aluminum concentration (eight different values ranging between 3.0×10^{-6} M to 3×10^{-3} M) were used. All steady-state determinations collected in a 10×8 matrix could be globally analyzed using Eq. 2 by a non-linear least-squares algorithm. The best-fit estimates for the parameters were obtained by extensive search on the parameter space using different starting guesses. Convergence to a single minimum was always obtained. Confidence intervals on the parameters were computed by F-testing at the cut-off of 1 standard deviation (68%).

The value of $K_{S,app}$ appears to be dependent on aluminum concentration (see Fig. 3) (thus $K^0_{S1}>K^0_{S0}$ and $K^{ES}_{Al}>K^{E0}_{Al}$), meaning that the substrate binding facilitates aluminum binding to its interaction cleft and vice versa. This behavior underlies a positive interaction network between aluminum binding pocket and substrate primary specificity site. This interaction appears to be pH independent for k_{cat} and $\alpha' k_{cat}$ (data not shown), indicating that protonation equilibria do not affect the catalytic events. On the other hand, $K_{S,app}$ is pH dependent (see Fig. 3). Analysis of the aluminum concentration effect on $K_{S,app}$ at every pH value, has been carried out according to the linkage theory [22], employing the equation reported above (Eq. 1), and in Fig. 4 the pH dependence is reported for the three parameters $K^0_{S,app}$, $K^{E0}_{Al,app}$ and $K^{ES}_{Al,app}$. The values of K_S and K^{E0}_{Al} are very similar [$K_S=3.2\ (\pm0.7)\times10^4\ M^{-1}$ and $K^{E0}_{Al}=3.6\ (\pm0.8)\times10^4\ M^{-1}$], the latter being close to that observed by fluorescence experiments (see Fig. 1B) and apparently pH independent. Such an observation indicates that: (1) $K^0_{S0}=K^{H1}_{S0}=K^{H2}_{S0}$ and (2) $K^{E0}_{Al}=K^{H1}_{Al}=K^{H2}_{Al}$, from which it follows that $K^{E0}_{H1}=K^{E1}_{H1}=K^{E0S}_{H1}$ and $K^{E0}_{H2}=K^{E1}_{H2}=K^{E0S}_{H2}$.

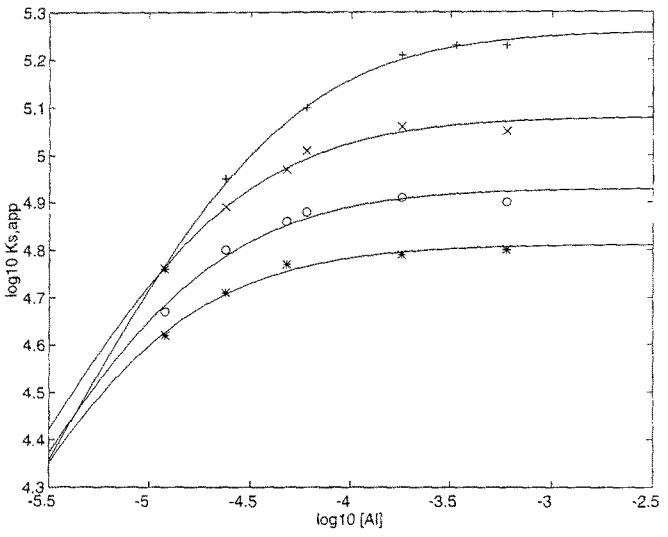


Fig. 3 Effect of increasing concentrations of aluminum on substrate recognition by α-chymotrypsin. Experiments were performed as reported in Materials and methods in 50 mM Mops-NaOH buffer at pH 6.0 (×–×); pH 6.5 (O–O); pH 7.4 (+–+) and pH 7.9 (*–*)

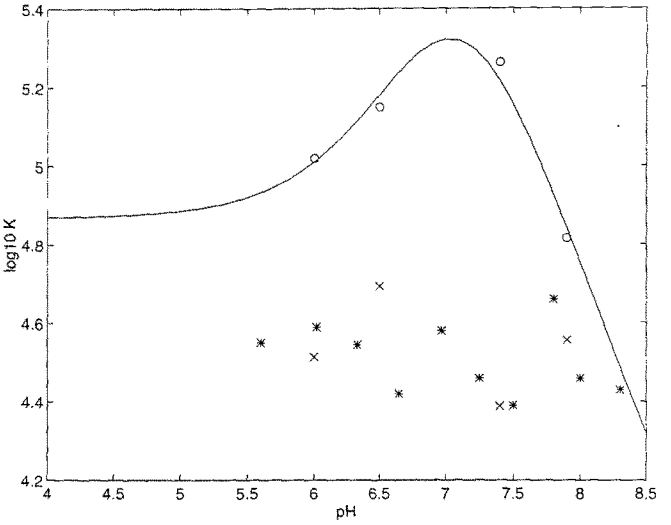


Fig. 4 Effect of pH on the $K^0_{S,app}$ (*–*), $K^{E0}_{Al,app}$ (×–×) and $K^{ES}_{Al,app}$ (O–O) values. Experiments were performed as reported in Materials and methods under the conditions reported in Fig. 3. The experimental data were analyzed according to Eq. 3

The lack of a pH effect on K^0_S and K^{E0}_{Al} (see also Fig. 4) indicates that neither binding of substrate alone nor the interaction of aluminum alone induces any relevant ligand-linked conformational change in the bound site. However, the occupancy of either one of the two sites (i.e. of the substrate and/or of aluminum) brings about a conformational change in the other binding pocket, with an alteration of the pK_a of functionally relevant groups. As a matter of fact, $K^{ES}_{Al,app}$ (and then K^0_{St}) displays a complex proton linkage, being characterized by a bell-shaped pattern (Fig. 4) which suggests that at least two protonation events modulate this parameter. The pH dependence of the equilibrium constant on two proton-linked equilibria can be described by the following equation:

$$K^{ES}_{Al,app}=K^{ES}_{Al}\frac{(1+K^{E1S}_{H1}[H^+]+K^{E1S}_{H1}K^{E1S}_{H2}[H^+]^2)}{(1+K^{E0S}_{H1}[H^+]+K^{E0S}_{H1}K^{E0S}_{H2}[H^+]^2)} \qquad (3)$$

where K^{E1S}_{H1}, K^{E1S}_{H2}, K^{E0S}_{H1} and K^{E0S}_{H2} are the proton binding equilibrium constants for the first and the second protonation event in the product and in the reactant species, respectively (see Scheme 1). Analysis of the pH dependence of K^{ES}_{Al} data (represented in Fig. 4) according to Eq. 3 allows us to determine the values of $\log K^{E1S}_{H1}=9.2\pm0.4$ and $\log K^{E1S}_{H2}=6.4\pm0.3$, and, in addition, of $\log K^{E0S}_{H1}$ ($=\log K^{E0}_{H1}=\log K^{E1}_{H1}$, see above)$=6.8\pm0.3$ and of $\log K^{E0S}_{H2}$ ($=\log K^{E0}_{H2}=\log K^{E1}_{H2}$, see

above) = 7.5 ± 0.4. We can thus fully describe, from a quantitative standpoint, the minimum scheme for ligand- and proton-linked equilibria (see Scheme 1). From this analysis it emerges that in α-chymotrypsin the interaction between substrate and aluminum-binding sites is regulated by at least two proton titrations, the first one displaying a pK of ca. 6.8. Such a group raises its pK to 9.2 upon binding of aluminum only in the presence of substrate, suggesting that the simultaneous presence of aluminum and substrate brings about a microenvironmental situation which renders that group more accessible to protonation. Furthermore, even in the absence of aluminum, the protonation of this group induces a conformational change of the enzyme, followed by the increase in pK of a second group up to 7.5; this phenomenon appears to be intrinsically related to the protonation equilibria of the protein itself. Upon aluminum and substrate binding, the pK of this group is lowered to 6.4, accounting for the bell-shaped pH-dependent behaviour reported in Fig. 4. Therefore, the interaction of one ligand, when the other binding pocket is occupied, increases the pK of one group by ~2.5 pH units, while for the second the pK is reduced by ca. 1 pH unit. This evidence suggests the presence of two ionizable groups, even though the two pKa values (7.5 and 6.8) can be considered distinguishable only at 67% s.c. Taken together, these data indicate the existence of two binding clefts whose protonation properties change according to whether only one is occupied or they are both saturated. A structural localization and interpretation of these features is difficult at this stage, even though the behavior of the second group might be reasonably referred to a histidine residue, which is a well-known aluminum chelating residue in proteins [23]. On the basis of Scheme 1, we can only propose that the occupancy of the substrate site brings about a ligand-linked conformational change which better shapes the interaction cleft for the aluminum ion. The structural modification most likely occurs at the ionic environment level through a partial masking of some positive charges, and this facilitates the formation of a salt bridge, raising their pKa values.

Concluding remarks

The results reported in this paper show that aluminum strongly influences the binding properties of α-chymotrypsin, affecting both the substrate and the inhibitor(s) recognition process. The linkage approach utilized for the analysis of the experimental data clearly indicates that the modulative effect is due to a simultaneous occupancy of the substrate and metal-binding sites and that the substrate binding brings about a modification of the ionic environment of the enzyme molecule, favoring aluminum binding.

Thus, it appears that the modulative effect exerted by the metal on chymotrypsin-like proteolytic activities is mediated by a concerted series of events which involve the metal and substrate (as well as inhibitor) binding and protonation phenomena occurring on the enzyme molecule. The "in vivo" significance of this finding can be accounted for by the potential role ascribed to chymotrypsin-like proteinases in brain metabolism, with particular reference to the etiology of Alzheimer's disease [24, 25] and to the debated role of aluminum in the deregulation of the brain proteolytic processes, leading to the generation of the senile plaques which is the major hallmark of this neurological disorder.

The data reported in this paper, as well as those previously obtained by Clauberg and Joshy [7], indicate that aluminum produces an overall activating effect on proteolytic enzymes which might result in an increase of the cerebral amount of β-amyloid peptides. This effect can be observed at physiological concentration of aluminum (estimated to be about 10 μM), reinforcing the idea of a key role for the metal in the mechanisms of plaque formation. However, a direct translation "in vivo" of the results reported here appears questionable both for the experimental conditions utilized for this study (i.e. the use of synthetic low-molecular-weight substrates for detecting the enzyme activity) and the inability to take into account the influence of other cellular effectors (such as chelating agents) which may counterbalance the metal effect. The important role of pH, evidenced in this study, in modulating the accessibility and the binding of aluminum on proteolytic enzymes appears also very interesting since it is well known that the bioavailability of aluminum is strongly influenced by the pH and that some degenerative phenomena occurring at cerebral level (with particular reference to Alzheimer's disease) are accompanied by changes of the pH value in some brain regions [26]. In addition, it has recently been reported that aluminum binding to β-amyloid peptides is strongly stabilized by slightly acidic conditions, which in turn favour also aggregation and precipitation [27] of β-amyloid peptides. Thus, an aluminum role in the pathogenesis of Alzheimer's disease could be envisaged at different levels. Firstly, the metal could affect the formation of the amyloid peptides through a positive modulation of the proteolytic processes involved in the β-amyloid precursor processing; then it could bind the so-formed peptides, promoting their aggregation, and precipitation could accelerate the neuritic plaque formation. Furthermore, a recent study [28] seems to support the idea that the interaction of proteinases with macromolecular inhibitors might be involved in the formation and clearance of β-amyloid peptides. Therefore, the negative heterotropic effect exerted by aluminum on the BPTI interaction with α-chymotrypsin suggests that aluminum binding induces a conformational change of the overall enzyme molecule, facilitating the interaction at the substrate primary specificity site where the synthetic substrate binds, but decreasing the overall free energy of the secondary specificity site where only BPTI interacts, as proposed by recent structural observations [29].

Taken together, the evidence reported in this paper indicates that, even though most likely several metabolic and genetic factors concur in the pathogenesis of Alzheimer's disease, the allosteric effect exerted by aluminum on proteolytic enzymes could play an important role, affecting many of the the early events involved in the onset of this pathology.

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