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Archives of Biochemistry and Biophysics 449 (2006) 157–163
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Conformational changes of bovine β-trypsin and trypsinogen induced by divalent ions: An energy-dispersive X-ray diffraction and functional study

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Received 16 December 2005, and in revised form 13 February 2006
Available online 6 March 2006

Abstract

The radius of gyration (R_g) of bovine trypsinogen and β-trypsin was measured by an energy-dispersive X-ray technique as a function of Ca^{2+} or SO_4^{2-} concentration; these results have been supplemented with measurements of association equilibrium constants of Ca^{2+} to its binding site(s) on both serine proteases and some of their adducts (with an effector and/or an inhibitor). As a whole, all information reported in the present work demonstrates that: (i) the strains exerted by different ions on these proteases produce diverse structural modifications; and (ii) at least in the case of Ca^{2+}, the changes in R_g can be ascribed to the direct interaction of the binding site(s) on the protein matrix with the cation.

Keywords: β-Trypsin; Trypsinogen; Proteases; Radius of gyration; Small angle scattering; X-ray diffraction; Structure

Nearly all proteins are built from domains, whose relative movements—especially when large—provide impressive examples of their structural flexibility. Such motions are important for a variety of protein functions, including catalysis, transport, and assemblies. Usually, the presence of bound ligands stabilizes a closed conformation, and their absence favors an open one. If this is how things stand, the interactions of proteins with small ligands is expected to embody some fundamental physico-chemical principles that are operative in relevant physiological regulation (e.g., enzyme activity and association among diverse macromolecules). Briefly, the study of interactions between proteins and small ligands furnishes the basis for the understanding of biological specificity at molecular level. Therefore, a deep insight into conformational changes of proteins in solution—as modulated by different solvent components—is an essential aspect for the comprehensive characterization of the overall structure and function of those macromolecules.

Among the most versatile tools for investigating the bulk properties of macromolecular solutions are scattering methods. In fact, despite of the low resolution of structural information obtained by small angle X-ray scattering techniques, their main advantage is the capability to provide direct information on the global shape and size of biological macromolecules in solution. More recently, an energy-dispersive X-ray diffraction (EDXD)[1] technique [1] has been shown [2]: (i) to be a very attractive way to measure the change in the overall dimensions (e.g., the radius of gyration, R_g) of biological systems, such as β-trypsin and

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[1] *Abbreviations used:* R_g, radius of gyration; EDXD, energy-dispersive X-ray diffraction; PSTI, pancreatic secretory tripsin inhibitor.

0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.02.010

trypsinogen, prominent members of the serine protease family; and (ii) not to induce structural modifications and/or damage during the data acquisition. Both β-trypsin and trypsinogen show an identical three-dimensional structure for about 85% of the polypeptide chain, but the rest of molecule (the so-called activation domain; see Fig. 1) is entirely different, being not observed in the electron density map of trypsinogen but visible in that of β-trypsin [3]. The physiological transition from inactive trypsinogen (i.e., the proenzyme or zymogen) to catalytically operative trypsin (i.e., the active enzyme) occurs by cleavage of an amino-terminal hexapeptide followed by the insertion of the newly formed amino-terminal residues (i.e., Ile16-Val17) into a specific cleft (the so-called Ile-Val pocket; see Fig. 1) present on the molecular surface. These events lead to the rigidification of the activation domain, an occurrence considered a clearcut evidence for the coupling mechanism between the activation domain and the reactivity center of the (pro)enzyme; in fact, the low intrinsic proteolytic activity as well as the weak ligand binding properties of the proenzyme may be related to the poorly structured recognition subsites (located in the reactivity or catalytic center; see Fig. 1) in comparison with those of the active enzyme. All these structural rearrangements can be properly induced in trypsinogen also by the binding of (i) activating peptides mimicking the amino-terminal residues of β-trypsin (e.g., the Ile-Val dipeptide), to the Ile-Val pocket, and (ii) a macromolecular inhibitor (e.g., pancreatic secretory trypsin inhibitor, or PSTI) that forms a stable complex interacting with the reactivity center of the proenzyme. In other words, the binding energy of each ligand (e.g., Ile-Val or PSTI) brings about a different activation state in trypsinogen that is not fully equivalent to that of β-trypsin; only the ternary complex PSTI:trypsinogen:Ile-Val shows the conformation of the activation domain very close to that of β-trypsin [3] and, accordingly, the overall binding energy (i.e., that due to the interaction between trypsinogen and PSTI plus that derived from the association between trypsinogen and Ile-Val)—propagating through the trypsinogen molecule—brings about a rigidification of the proenzyme even though not fully equivalent (see legend to Fig. 1) to that produced by the endogeneous amino-terminus of the enzyme.

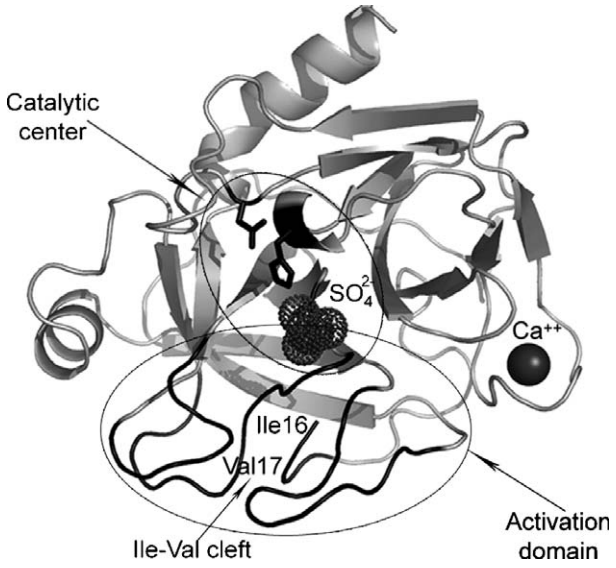

Fig. 1. Richardson diagram of trypsin(ogen), lacking the amino-terminal hexapeptide that protrudes from the polypeptide end present in the Ile-Val cleft. The main structural characteristics of such protease(s) are shown. In the middle of the drawing, the catalytic center is depicted: it comprehends (i) catalytic residues drawn (two out of the relevant three, i.e., His57 and Asp102, the third one—Ser195—being hidden by SO_4^{2-}) in ball-and-stick representation; (ii) one SO_4^{2-} anion; and (iii) the recognition and binding sites for substrates and inhibitors (not portrayed). At the bottom, four polypeptide loops that are not visible in the Fourier map of trypsinogen are shown (three of these segment build up the so-called activation domain; the fourth segment assumes an ordered three-dimensional structure only when trypsinogen is converted into trypsin by specific cleavage of the amino-terminal hexapeptide and insertion of the newly formed terminus into the Ile-Val cleft).

In a previous investigation on the same biological systems [2] the strains exerted on both proteins, and reflected in modifications of their overall shape (as measured by changes in R_g), by chloride ion and by protons were determined; however, the topology of the binding sites of these ions on the proteins was not known, and as a consequence the interpretation was only phenomenological. Therefore, to extend this previous study and to analyse in more detail the interaction of biologically important ions with the trypsinogen/β-trypsin system, the effect of one cation (calcium ion, Ca^{2+}) and one anion (sulphate ion, SO_4^{2-}), whose location is well established on these serine proteases [4–7], on the R_g values of both proteins has been investigated by using the EDXD technique; moreover, these results have been supplemented with measurements of association equilibrium constants of Ca^{2+} to its binding site(s) on both serine proteases and some of their adducts. As a whole, all information, collected under equilibrium conditions, demonstrates that (i) although spatially separated, the three binding pockets considered (i.e., the Ile-Val cleft, the reactive center where PSTI binds and the Ca^{2+} loop) are cooperatively linked, and (ii) at least in the case of Ca^{2+}, the changes in R_g can be ascribed to the direct interaction of the protein matrix with the cation.

Materials and methods

Materials

Bovine trypsin was purchased from Sigma Italia (Milan, Italy). After purification of the commercial product, achieved according to a previous procedure [8] as modified by Luthy et al. [9] β-trypsin preparations contained less than 1% of α-trypsin, as judged from kinetics of the reaction with *p*-nitrophenyl *p*-guanidinobenzoate [10]. Crystalline trypsinogen was obtained from Merck (Darmstadt, Germany); zymogen preparations were found virtually free of trypsin, chymotrypsin, elastase, and kallikrein activities [11].

The homogeneity of β-trypsin and trypsinogen was checked by polyacrylamide gel electrophoresis in 1% sodium dodecylsulfate, in the presence and absence of 1% mercaptoethanol [12]. The preparations used contained less than 1% of protein contaminants.

Bovine PSTI was prepared and purified as previously described [13].

The Ile-Val dipeptide was synthesized as detailed in [14].

The concentration of β-trypsin was determined: (i) spectrophotometrically at 280 nm [$\varepsilon(1\%,\ 1\ \text{cm}) = 15.6$ at pH 6.80, phosphate buffer, $I/2 = 0.1$ M, and $T = 20$ °C]; and (ii) by titration with pNGB [10]. Trypsinogen concentration was measured (i) by titration with pNGB and (ii) spectrophotometrically at 280 nm [$\varepsilon(1\%,\ 1\ \text{cm}) = 13.9$]. The values of the enzymes' concentrations, determined by the two different methods, agree very well with one another (the difference being less than 5%). PSTI concentration was obtained using $\varepsilon(1\%,\ 1\ \text{cm}) = 5.18$ [15].

Benzamidine, obtained from Sigma (St. Louis, USA), was of analytical grade and used without further purifications; its concentration was determined spectrophotometrically at 230 nm [$\varepsilon(1\ \text{mM},\ 1\ \text{cm}) = 12.7$]. All other chemicals were from Carlo Erba (Milan, Italy).

Sample preparation for EDXD experiments

When the protein solution is diluted, small angle X-ray scattering measurements are affected by interparticular interference effects, a phenomenon that can be eliminated by extrapolation to zero concentration. Since no difference in the scattering curves was observed to occur between 4 and 10 mg/mL [2], all samples for EDXD experiments were prepared at a protein concentration of 7 mg/mL (~0.3 mM) in order (i) to avoid any important interaction between protein molecules, and (ii) to collect scattered intensity statistically meaningful. To prevent any (auto)catalytic effect of β-trypsin or its action on trypsinogen during the time of data acquisition, 50 mM benzamidine was added to the solvent [2]. The $CaCl_2$ profile was investigated in 50 mM Bis-Tris, pH 6.5, while that of Na_2SO_4 in 0.1 M sodium acetate buffer, pH 4. All experiments were carried out at room temperature.

EDXD measurements

Small angle X-ray scattering experiments were performed by using two energy-dispersive X-ray diffractometers extensively described elsewhere [1,16]. The effect of Ca^{2+} on the spatial form of β-trypsin and trypsinogen and that of SO_4^{2-} on trypsinogen shape were investigated by using the apparatus located at the Physics Department, whereas the influence of SO_4^{2-} on both β-trypsin and trypsinogen conformational changes as well as that of Ca^{2+} on β-trypsin overall shape were studied by means of the instrumentation at the Chemistry Department. All the replicated experimental measurements gave identical results, thus providing an important confirm on the superimposition of the scattering curves by the two different apparatuses.

Both devices consist of a Seifert X-ray generator supply, a water-cooled tungsten X-ray source whose *bremsstrahlung* radiation is used. The operating conditions were: 45 mA current intensity, 50 kV high voltage supply, 2250 W total power and 11–50 keV energy range. For acquiring the small angle X-ray scattering spectra, a Ge solid-state detector (SSD) connected via an electronic chain to a multi-channel analyzer was used. A set of four collimating W slits placed in front and behind the sample complete the diffractometer operating in a vertical θ/θ transmission geometry. Selecting two scattering angles $\theta = 0.15°$ and $\theta = 0.25°$ the s range (s is the momentum transfer defined as $s = 2\ \sin\theta/\lambda$) was 0.0042–0.045 Å^{-1}. Both trypsin and trypsinogen samples were measured at room temperature in a sample holder with mylar windows placed in the optical center of the diffractometer. The exposure time for one measurement was typically 20,000 s. Because of the low X-ray dose to which the samples were exposed as EDXD experiments proceeded, neither specific nor aspecific damages occurred. The measured experimental intensities were normalized to the incident radiation intensity and corrected for buffer contributions, parasitic contributions due to mylar windows, and sample transmission.

Data analysis

Theoretical background

A monodisperse solution of biological macromolecules of constant electron density ρ, embedded in a solvent of constant ρ_0, is an ideal two-phase model where only the excess $\Delta\rho = \rho - \rho_0$ is relevant for the scattering.

X-ray scattering by proteins in solution is sensitive to spatial dishomogeneities in electron density. Solutions are isotropic samples: the particles in brownian motion act as randomly oriented with respect to the direction of the incident beam and only the spherical average of the excess X-ray scattering intensity is accessible [17].

$$I(s) = \langle I(\vec{s})\rangle_{\Omega}, \tag{1}$$

where Ω is the solid angle in the reciprocal space. The scattered intensity can be expanded in powers of s^2 and at small s, the scattering pattern can be approximated by a Gaussian [18]

$$I(s) \cong I(0)\exp\left(-\frac{4\pi R_g^2 s^2}{3}\right) \tag{2}$$

whose width is proportional to the square of the gyration radius of the particle, R_g, defined for a homogeneous scattering particle by

$$R_g^2 = \frac{1}{2}\int_0^{\infty} p(r) r^2 \mathrm{d}r, \tag{3}$$

where $p(r)$ is the pair distribution function defined as

$$p(r) = \frac{1}{\pi}\int_0^{\infty} rsI(s)\sin(2\pi rs)\mathrm{d}s \quad (4)$$

Binding measurements

Calcium binding constants were measured at 20 °C with a calcium-selective electrode equipped with a membrane that was freshly prepared according to the method elsewhere described [19]. The proteins were dissolved in deionized water and brought to pH 6.4–6.6 with 1 M NaOH or HCl. The calcium content of the proteins as well as that of the $CaCl_2$ stock solution was determined by atomic absorption spectroscopy.

Results

To minimize side effects caused by some of the ions co-present in the solvent, different pH values were chosen when the influence of the two salts, i.e., $CaCl_2$ and Na_2SO_4, on the R_g has been measured. With reference to such a choice, it is useful to remind the readers that the pH profile of the R_g corresponds to a bell-shaped curve for both proteinases investigated, that of trypsinogen being shifted along the ordinate axis by ~1 Å [2]; in particular a narrow peak in the R_g values is centered at pH ~ 6.5, the left and right branches of the curve levelling off from, pH 5.5, towards lower values and from, pH 7.5 up, respectively.

The effect of $CaCl_2$ on the radius of gyration (R_g) of both β-trypsin and trypsinogen has been evaluated at pH 6.5 (i.e., at the highest pH effect on R_g), a value corresponding to a proton concentration that promotes the binding of Ca^{2+} and restricts the association of Cl^-; in fact, evidence based on Cl^- determination by a permselective membrane electrode [20] has demonstrated that just at pH 6.5 the number of Cl^- bound to serine proteases drops to a minimum. The R_g of β-trypsin, directly calculated from Eq. (2), presents its highest value ($R_g \sim 22.5$ Å) in the absence of Ca^{2+} and reaches its lowest magnitude ($R_g \sim 20.5$ Å) at ~20 mM of metal ion (Fig. 2). By increasing Ca^{2+} concentration no further structural modifications can be observed (at least within the sensitivity limits of the instrument) and accordingly the value of R_g achieved at 20 mM remains constant up to 300 mM $CaCl_2$. On the other hand, in the case of trypsinogen, the values of R_g actually go through a minimum that corresponds to 20.4 Å at ~10 mM $CaCl_2$; successive addition of Ca^{2+} produces an increase in trypsinogen R_g until a plateau is reached corresponding to a value of 22.5 Å at ~150 mM $CaCl_2$ (Fig. 3). The larger value in R_g (~1 Å) measured for trypsinogen relative to β-trypsin (under similar solvent conditions, i.e., in the absence of Ca^{2+}; see Figs. 2 and 3) can easily be explained in terms of the presence of an extended amino-terminal hexapeptide in trypsinogen that is removed in β-trypsin (see Introduction). Moreover, it is interesting to note that the reference R_g values for both proteases (i.e., the R_g magnitude when sticky ions, such as Ca^{2+}, are not in the solvent) are significantly greater in this work (see Figs. 2 and 3) with respect to those previously reported [2,21]. This evidence however is not unexpected, since just around, pH 6.3 [i.e., very close to the experimental conditions chosen in this work, pH 6.5, and different from the previous ones (pH 4)] the effect of protons on the R_g is the highest for both β-trypsin and trypsinogen [2], and in addition, in some of the other works,e.g. [21], solvent components such as borate, citrate or phosphate—all able to bind the proteases—were present. Moreover, the contribution of the counter-ion (i.e., Cl^-) to the measured (at pH 6.5) effect on the R_g of both β-trypsin and trypsinogen is expected to be negligible, and not only for the low affinity of this anion [20] but also since the profiles of the curves obtained in the presence of $CaCl_2$ (see Figs. 2 and 3) are mirror images relative to those determined for Cl^-, see [2]; in other words, any Cl^- binding should reduce the R_g induced by Ca^{2+} and therefore the observed magnitude of R_g in $CaCl_2$ may be considered to correspond to its minimum value.

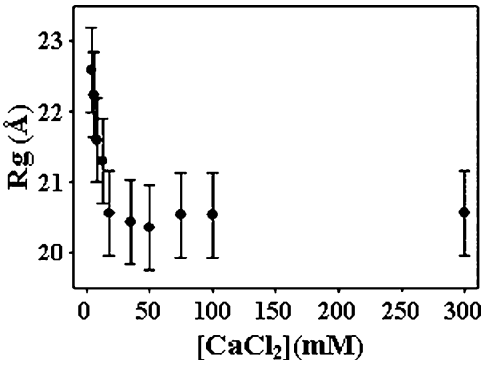

Fig. 2. Profile of the radius of gyration (R_g) of β-trypsin (7 mg/ml, pH 6.5, and room temperature) as a function of Ca^{2+} concentration. The values are calculated by the Guinier approximation (see Eq. (2) in the text).

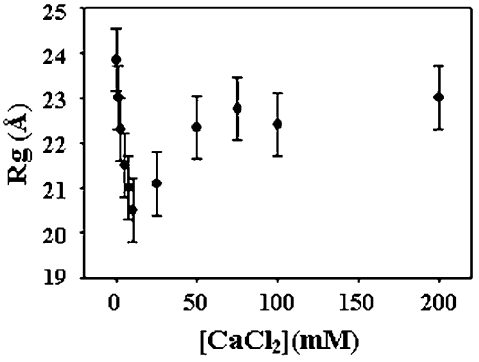

Fig. 3. Profile of the radius of gyration (R_g) of bovine trypsinogen (7 mg/ml, pH 6.5, and room temperature) as a function of Ca^{2+} concentration.

Estimations of the equilibrium binding constants (K) for the high affinity Ca^{2+} association site have been attempted by several groups [22–27]; however, the reported variation in the K values differ from one another by more than one order of magnitude because of the various experimental conditions and the diverse methods used. Therefore, to accurately evaluate in quantitative terms the coupling between the Ca^{2+} association to both proteases and their

large conformational changes (i.e., those changes related to modifications in macromolecule size and shape, as measured by R_g), the equilibrium binding constants of Ca^{2+} with trypsinogen, its binary (with Ile-Val or PSTI) as well as ternary (with Ile-Val and PSTI) adducts and—for comparative reasons—with β-trypsin were determined, under the experimental conditions chosen for the scattering measurements. The association equilibrium constants for the protein systems investigated are summarized in Table 1. A significant influence of all ligands (i.e., Ile-Val, and PSTI) on the Ca^{2+} affinity is evident, suggesting the existence of a structural coupling between the Ca^{2+}binding loop and other sites on the protease surface for associating effectors (e.g., the Ile-Val dipeptide) and/or inhibitors (e.g., PSTI); these energy routes are spread over most of the protein matrix (see also Fig. 1) and are active under the very same conditions used for the EDXD experiments.

In the case of Na_2SO_4 (whose experiments were carried out at pH 4, i.e., at the minimum pH effect on R_g, to favor the interaction of the anion (i.e., SO_4^{2-} by keeping the protein positively charged, and at the same time to prevent the Na^+ association, as demonstrated by [20], the R_g values for β-trypsin and trypsinogen actually go through a maximum (Figs. 4 and 5) that correspond to 20.5 Å for trypsin and 22.8 Å for trypsinogen, at 50 mM and 60 mM Na_2SO_4, respectively: these values of R_g rapidly decrease on both abscissa sides until limit values are reached (e.g., 16.5 and 17.2 Å for trypsin and trypsinogen, respectively, on the right branch of the curves). Such bell-shaped curves suggest the existence of two (classes of) binding sites for SO_4^{2-}

Table 1
Association equilibrium constants characterizing the Ca^{2+} binding to bovine β-trypsin and trypsinogen (the latter in the free form and as an adduct with PSTI and/or Ile-Val) (experimental conditions: 50 mM Bis-Tris, pH 6.5, at 20 °C; protein concentration: 7 mg/mL. An average error value of ±8% was ascribed to K values, as the standard deviation)

System	K (M^{-1})
β-Trypsin	3.4×10^4
trypsinogen	5.9×10^3
Ile-Val:trypsinogen	7.8×10^3
Trypsinogen:PSTI	2.7×10^4
Ile-Val:trypsinogen:PSTI	3.0×10^4

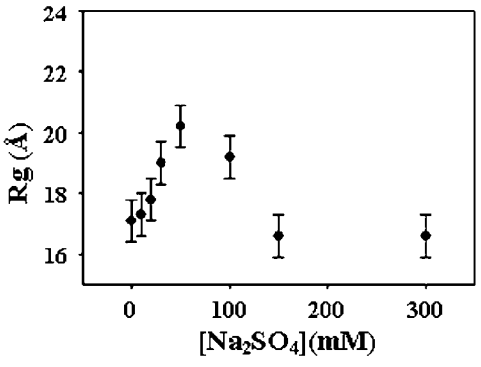

Fig. 4. Profile of the radius of gyration (R_g) of β-trypsin (7 mg/ml, pH 4, and room temperature) as a function of SO_4^{2-} concentration.

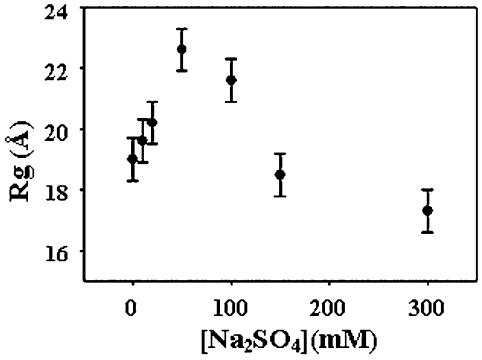

Fig. 5. Profile of the radius of gyration (R_g) of trypsinogen (7 mg/ml, pH 4, and room temperature) as a function of SO_4^{2-} concentration.

possibly very similar in structural terms for both proteases but with different ion affinity. The slight shift of the trypsinogen curve relative to that of β-trypsin along the Cartesian axes finds a simple explanation in the following considerations: (i) the displacement along the ordinate axis is forced by the presence of a six residues long tail in trypsinogen; and (ii) that along the abscissa axis is expected because a fraction of the intrinsic binding energy of SO_4^{2-} is spent in shaping disordered region(s) of trypsinogen (a molecule more flexible than β-trypsin).

Discussion

Six SO_4^{2-} anions have been found on the bovine β-trypsin surface [6,7,28], one located at the reactive center (see Fig. 1), and the remainder five bound on other portions of the molecular surface. All SO_4^{2-} anions (i) form salt bridges or hydrogen bonds with protein atoms or water molecules, and (ii) impose the conformation of the residue side chains near them to change greatly [7]. Since all amino acid residues constituting the binding sites for SO_4^{2-} in β-trypsin are outside of the activation domain [7], only differences in affinity and not in the number of SO_4^{2-} bound to trypsinogen are expected. Such a prospect is in good agreement with values of R_g determined for both proteases (see Figs. 4 and 5). In particular, the left branch of both bell-shaped curves may tentatively be assigned to the effect of the only SO_4^{2-} in the active site of the two proteases, which is the one with the highest affinity; and, therefore, the influence of the remainder five SO_4^{2-} per macromolecule on the overall shape of these proteins is described by the right branch of the curves whose slopes are different in the two cases: while for β-trypsin the slant is steeper even though not perfectly steady, in the case of trypsinogen the descent is smoother and reaches the lowest value of R_g at a concentration of SO_4^{2-} twice that of β-trypsin (150 mM vs 300 mM; see Figs. 4 and 5). This means that, as expected, the low affinity class of SO_4^{2-} binding sites appears to be much more homogeneous in the more compact protein (i.e., β-trypsin) with respect to the flexible trypsinogen; thus, in the latter case the strains exerted by SO_4^{2-} anions on the protein matrix are expected to be confined to the

proximity of the binding site and therefore each individual ligand association should retain its own energetic characteristics. An alternative explanation for the physical mechanism (other than specific ion binding) by which SO_4^{2-} concentration (>100 mM; see Figs. 4 and 5) can affect the overall shape of the two proteinases, could be the Hofmeister effect [29,30]. According to this viewpoint, strongly hydrated anions, such as SO_4^{2-}, are expected [31] to interact only slightly with peptide bonds [32] in contrast with weakly hydrated anions, such as Cl^-. Therefore, Cl^- binding to, e.g., trypsinogen at concentrations>100 mM is expected to be stronger than SO_4^{2-}, a behaviour that hardly corresponds to the observed one (see Fig. 5 of the present work vs. Fig. 8 in [2]). As a whole, the overall effect of SO_4^{2-} at higher concentrations on the R_g values is not unequivocal in its molecular mechanism and may represent the consequence of three actions: direct anion binding, the Hofmeister effect and Debye screening.

More information at molecular level can be extracted from the experiments on Ca^{2+}. It is well known that this cation, among other functions, accelerates the conversion of trypsinogen to β-trypsin, protects the active enzyme against autodigestion and markedly increases the thermal stability. A common feature of trypsinogen and β-trypsin is the presence of one Ca^{2+} binding site that has an affinity in the order of 10^3–10^4 (e.g., see Table 1) and is situated in a loop (see Fig. 1) comprising amino acid residues 70–80 [4]. In trypsinogen, there is an additional Ca^{2+} binding site of lower affinity ($K = 6.25 \times 10^2\ M^{-1}$; [24]), which has been assigned to the two aspartyl residues 13 and 14 (i.e., located at the amino-terminal hexapeptide of trypsinogen and absent in β-trypsin) neighbouring the important Lys15-Ile16 bond that is split during the activation of trypsinogen to β-trypsin. The high affinity site for Ca^{2+} has been identified by X-ray crystallography in both trypsinogen and β-trypsin [4,33]: Ca^{2+} is complexed by six different atomic groups positioned at the edges of an almost regular octahedron, in a loop located more than 21 Å far from the cleft where most of the reactive residues (such as Ser195 and His57) are arranged (see also Fig. 1). Moreover, nuclear magnetic resonance data [26] indicate that the Ca^{2+} binding site maintains the crystal structure symmetry also in solution. This high affinity binding site for Ca^{2+} in trypsinogen is completely saturated at 20 mM $CaCl_2$ [1], a value in line with the scattering (see Fig. 3) as well as the binding (see Table 1) results reported in the present work. In spite of the distance of the Ile-Val cleft from the Ca^{2+} loop (see Fig. 1), however, the binding of the dipeptide both to free trypsinogen and trypsinogen:PSTI binary complex influences (under the very same conditions used for the R_g determination) the configuration of the Ca^{2+} binding site, suggesting that in this case the conformational transitions of trypsinogen are regulated by the binding to diffusible structural units (such as Ca^{2+} or Ile-Val and/or PSTI). This evidence parallels the data obtained [27] with squash seed protease inhibitor(s), very different in terms of mass (28–32 amino acid residues) relative to PSTI (56 residues). Moreover, the equilibrium association constant of the squash inhibitor with bovine trypsinogen increases 3-fold in the presence of 20 mM $CaCl_2$ [27], a result in line with the data obtained in this study with PSTI.

An additional, central feature of importance in the mechanism of such propagation of chemical information, which is effected mechanically by strains set up in a structure held together only by secondary bonds, is that the Ca^{2+} binding energy spreads from its association site to other portions of the macromolecule without significant secondary structure perturbation, as measured by infrared spectroscopy [34]. In fact, there is no need to postulate any major structural rearrangements when the free energies of the numerous secondary interactions that support the internal conformation of these proteases and those that stabilize the association of ligands with their binding sites in proteins, are of the same order of magnitude. However, the reported data give no information about the molecular mechanism with which the transmission of information from one portion (e.g., the Ca^{2+} loop) of the protease to another (e.g., the reactive center) can be visualized; that is: (i) the ligand (Ca^{2+}) binding simply shifts the equilibrium of an ensemble of subsites, so that the bound conformation becomes the center of a new, possibly narrower distribution, or (ii) the ligand binding acts on the protease, imagined as a globule of plastic material capable of flowing under an applied stress, by strains and distorsions that become operative only after a *yield value* has been reached, just as in the case for a non-Newtonian, or Maxwellian fluid [35].

In conclusion, the reported results provide experimental evidence that the conformational changes observed in trypsin(ogen) (i) strongly depend on the specific ion present in the solvent, and (ii) are governed—at least in the case of Ca^{2+}—by direct ion binding; in this latter case, in fact, the R_g magnitude reaches a constant value (see Figs. 2 and 3) when Ca^{2+} saturates its binding site(s) on the protease (see Table 1), i.e., when Ca^{2+} concentration corresponds to $\sim 100 \times (1/K)$ (as also observed for the second Ca^{2+} binding site whose profile slope (see Fig. 3) approaches zero in trypsinogen at ~150 mM). Since different ions possess different binding sites and exert different strains (e.g., see Ca^{2+} vs SO_4^{2-}), many parts of the protein structure are expected to contribute towards the shift in energy and conformation taking place upon ligand binding; however, to give every part of the protein its precise weight in such process seems certainly beyond present day possibilities.

References

[1] R. Caminiti, C. Sadun, V. Rossi, F. Cillocco, L. Bencivenni, J. Macromol. Sci. Phys. 835 (1996) 199–213.
[2] G. Caracciolo, G. Amiconi, L. Bencivenni, G. Boumis, R. Caminiti, E. Finocchiaro, B. Maras, C. Paolinelli, A. Congiu Castellano, Eur. Biophys. J. 30 (2001) 163–170.
[3] R. Huber, W. Bode, Acc. Chem. Res. 11 (1978) 114–122.
[4] W. Bode, P. Schwager, J. Mol. Biol. 98 (1975) 693–717.

[5] J.B. Abita, M. Lazdunski, Biochem. Biophys. Res. Commun. 35 (1969) 707–712.
[6] H.D. Bartunik, L.J. Summers, H.H. Bartsch, J. Mol. Biol. 210 (1989) 813–828.
[7] G. Zhu, Q. Huang, Z. Wang, M. Qian, Y. Jia, Y. Tang, Biochim. Biophys. Acta 1429 (1998) 142–150.
[8] T. Schroeder Jr., E. Shaw, J. Biol. Chem. 243 (1968) 2943–2949.
[9] J.A. Luthy, M. Praissman, W.R. Finkenstadt, M. Laskowski Jr., J. Biol. Chem. 248 (1973) 1760–1771.
[10] J.F. Hruska, J.H. Low, F.J. Kézdy, Biochem. Biophys. Res. Commun. 36 (1969) 272–277.
[11] E. Antonini, P. Ascenzi, M. Bolognesi, M. Guarneri, E. Menegatti, G. Amiconi, Mol. Cell. Biol. 60 (1984) 163–181.
[12] K. Weber, J.R. Pringle, M. Osborn, Methods Enzymol. 26 (1972) 3–27.
[13] E. Menegatti, F.R. Bortolotti, L. Minchiotti, A. De Marco, Biochem. Biophys. Acta 707 (1982) 50–57.
[14] E. Antonini, P. Ascenzi, M. Bolognesi, G. Gatti, M. Guarneri, E. Menegatti, J. Mol. Biol. 165 (1983) 543–558.
[15] L.J. Greene, J.J. Di Carlo, A.J. Sussman, D.C. Bartelt, D.E. Roark, J. Biol. Chem. 243 (1968) 1804–1809.
[16] F. Boffi, R. Caminiti, C. Sadun, S. Capuani, A. Giovannelli, A. Congiu Castellano, Chem. Phys. Lett. 286 (1998) 473–478.
[17] P. Vachette, M. Koch, D.I. Svergun, Methods Enzymol. 374 (2003) 584–615.
[18] A. Guinier, G. Fournet, Small Angle Scattering of X-rays, Wiley, New York, 1955.
[19] J. Ruzicka, E.H. Hansen, J.C. Tjell, Anal. Chim. Acta 67 (1973) 155–175.
[20] F. Friedberg, S. Bose, Biochemistry 8 (1969) 2564–2567.
[21] S.J. Perkins, K.F. Smith, J.M. Kilpatrich, J.E. Valanakis, R.B. Sim, Biochem. J. 295 (1993) 87–99.
[22] M. Delaage, M. Lazdunsk, Biochem. Biophys. Res. Commun. 28 (1967) 390–394.
[23] M. Epstein, A. Levitzki, J. Reuben, Biochemistry 13 (1974) 1777–1782.
[24] E. Colomb, C. Figarella, Biochim. Biophys. Acta 571 (1979) 397–405.
[25] S.G.R. Cliffe, D.A.W. Grant, Biochem. J. 193 (1981) 655–658.
[26] E. Chiancone, T. Drakenberg, O. Teleman, S. Forsén, J. Mol. Biol. 185 (1985) 201–207.
[27] T. Zbyryt, J. Otlewski, Biol. Chem. 372 (1991) 255–262.
[28] S.O. Smith, S. Farr-Jones, R.G. Griffin, W.W. Bachovchin, Science 244 (1989) 961–964.
[29] R.B. Baldwin, Biophys. J. 71 (1996) 2056–2063.
[30] C.H.L. Ramos, R.L. Baldwin, Protein Sci. 11 (2002) 1771–1778.
[31] K.D. Collins, Methods 34 (2004) 300–311.
[32] P.K. Nandi, D.R. Robinson, J. Am. Chem. Soc. 94 (1972) 1308–1315.
[33] M. Bolognesi, G. Gatti, E. Menegatti, M. Guarneri, M. Marquart, E. Papamokos, R. Huber, J. Mol. Biol. 162 (1982) 839–868.
[34] R.A. Buono, S.J. Prestrelski, M.N. Liebman, D.M. Byler, Biochim. Biophys. Acta 1204 (1994) 124–128.
[35] R. Houwink (Ed.), Elasticity, Plasticity, and Structure of Matter, Dover, New York, 1958, p. 7.