Eur Biophys J (2001) 30: 163–170
DOI 10.1007/s002490000128

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Conformational study of proteins by SAXS and EDXD: the case of trypsin and trypsinogen

Received: 3 August 2000 / Revised version: 30 November 2000 / Accepted: 30 November 2000 / Published online: 6 March 2001

Abstract The radius of gyration (R_g) of bovine trypsinogen and β-trypsin was measured by an energy-dispersive X-ray technique (EDXD) and by small-angle X-ray scattering (SAXS), under different solvent conditions. Both techniques gave superimposable results. The experimental evidence demonstrated that: (1) no structural modifications and/or damage occurred during the data acquisition by EDXD; (2) at pH 4 the active enzyme has one class of chloride binding sites in common with the zymogen, whereas the latter protease shows an additional class able to reverse the effects on R_g induced by chloride at low concentration; and (3) the pH profile of the R_g of both proteases does not resemble at all the pH effect on β-trypsin activity, a result in line with the finding that the electrical potentials induced by surface charge are small in absolute magnitude and produce no gradient across the active site.

Keywords β-Trypsin · Trypsinogen · Small angle scattering · Diffraction

Introduction

Structural studies of macromolecules of biomedical interest are aimed at establishing a close relationship between their structure (or, more often, their structural changes) and mode of action. In the 1980s, X-ray crystallography and multidimensional NMR spectroscopy emerged as widespread and powerful tools for providing detailed information on positions in space (Cartesian coordinates) of individual atoms in biological macromolecules. However, these high-resolution methods apply under rather specific conditions: thus, it is often difficult to grow crystals of many proteins, and NMR studies are mostly limited to small macromolecules. Therefore, even though at only low and medium resolution, information on the overall shape of biological macromolecules over a wide range of particle sizes (~1 to ~100 nm in dimensions), as well as on the conformational responses to changes in the solution conditions, is often collected by small-angle scattering of X-rays (SAXS) (Glatter and Kratky 1982; Durchschlag et al. 1991; Congiu Castellano et al. 1994, 1996) or neutrons (Trewhella 1997). More recently, an energy-dispersive X-ray diffraction technique (EDXD) (Caminiti et al. 1996) seems to be a most attractive way to measure the change in the overall dimensions of biological systems (Boffi et al. 1998). The latter technique has many advantages (Caminiti and Rossi Albertini 1999) and some drawbacks relative to the other methods. Among the unfavorable characteristics of EDXD, the long acquisition time (~12 h) could constitute a real danger in that biological macromolecules might be damaged by X-ray irradiation, as already observed in X-ray crystallography (Blundell and Johnson 1976), even though the total dose absorbed by the samples in EDXD experiments is much smaller. Therefore, aided by tools useful for protein analysis (e.g. HPLC, electrophoresis, sequencer), the comparison of geometric properties of monodisperse identical particles in solution, as extracted from the same samples by EDXD and SAXS under various experimental conditions, appears to be of great interest.

Both SAXS and EDXD are in principle able to provide similar information about parameters such as the radius of gyration (R_g). Accordingly, in order to shed

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more light on the stability of biological macromolecules under lasting X-ray flux in EDXD experiments, some effects of solvent components on the overall protein dimensions, observed with both EDXD and SAXS apparatuses, of two monomeric proteins (trypsin and trypsinogen) in solution have been investigated under conditions differing in terms of pH and anion concentration. Trypsin is a prominent member of the serine protease family (Marquart et al. 1983) and forms the functional principle of many highly specific proteases involved in blood clotting and complement binding (Neurath 1999). These proteases have a trypsin-like core with large segments attached to it, which modify their molecular biorecognition properties. The pro-enzyme trypsinogen (229 amino acid residues) is converted to β-trypsin by cleaving the amino-terminal hexapeptide. The process of autolysis may continue in β-trypsin (Keil 1971) and the sequence position of several successive autolytic cleavages has been precisely determined. Thus, the cleavage of β-trypsin at the bond Lys131-Ser132 leads to α-trypsin, a two-chain structure held together by disulfide bonds; under different conditions, the bond Arg105-Val106 can also be cleaved without the loss of activity, and a further cleavage of α-trypsin at the bond Lys176-Asp177 yields another active form of trypsin, pseudo-trypsin or ψ-trypsin. The crystal structures of some serine proteases, such as porcine α-trypsin (Johnson et al. 1997), porcine ϵ-trypsin (Huang et al. 1994), porcine β-trypsin (Huang et al. 1993) and bovine β-trypsin (Bartunik et al. 1989), have been refined at high resolution (1.5–1.8 Å).

Both trypsinogen and β-trypsin have an identical three-dimensional structure for about 85% of the polypeptide chain, but the rest of the molecule (the so-called activation domain) is entirely different (Huber and Bode 1978). The transition from the inactive trypsinogen to catalytically active β-trypsin is triggered by the rigidification of the activation domain that is not observed in the electron-density map but is visible in β-trypsin. Most residues of the activation domain (35 out of 41) are immediately adjacent to the active site of β-trypsin, and their structural disorder accounts for the much reduced proteolytic activity of trypsinogen compared with β-trypsin (Ascenzi et al. 1988). Further crystallographic work at low temperature and molecular dynamics studies showed that the six amino-terminal residues of trypsinogen, cleaved on activation, were predominantly flexible (Perkins and Wüthrich 1980; Brunger et al. 1987); moreover, it was inferred that the remaining 35 residues in trypsinogen adopt a range of conformational substates, making the activation domain in this zymogen the most extensive disordered region documented to date in a relatively small protein. Since this is how things stand in structural terms for β-trypsin and trypsinogen, comparison of the changes in their radius of gyration, as induced by variations in solvent composition, appears to be of interest per se and can assist in revealing general features of solvent component binding to serine proteases.

Materials and methods

Bovine trypsin was purchased from Sigma Italia (Milan, Italy). After purification of the commercial product, achieved according to a previous procedure (Schroeder and Shaw 1968) as modified by Luthy et al. (1973), β-trypsin preparations contained less than 1% of α-trypsin, as judged from kinetics of the reaction with *p*-nitrophenyl *p*-guanidinobenzoate (pNGB) (Hruska et al. 1969). Crystalline trypsinogen was obtained from Merck (Darmstadt, Germany); zymogen preparations were found virtually free of trypsin, chymotrypsin, elastase and kallikrein activites (Antonini et al. 1984).

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 (Weber et al. 1972). The preparations used contained less than 1% of protein contaminants.

The concentration of β-trypsin was determined: (1) spectrophotometrically at 280 nm [ϵ (1%, 1 cm) = 15.6, at pH 6.80, phosphate buffer, $\Gamma/2 = 0.1$ M, and $T = 20$ °C]; and (2) by titration with pNGB (Hruska et al. 1969). Trypsinogen concentration was determined (1) by titration with pNGB and (2) spectrophotometrically at 280 nm [ϵ (1%, 1 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%).

Benzamidine, obtained from Sigma (St. Louis, USA), was of analytical grade and used without further purification; its concentration was determined spectrophotometrically at 230 nm [ϵ (1 mM, 1 cm) = 12.7]. All other chemicals were from Carlo Erba (Milan, Italy). *N*-Benzyloxycarbonylarginine *p*-nitrophenyl ester (Z-ArgONp) was synthesized according to a previous procedure (Glass and Pelzig 1978).

Small-angle X-ray scattering experiments

SAXS data were collected at the experimental station D24 of the D.C.I. storage ring of LURE (Laboratoires pour l'utilization de la radiation electromagnetique) in Orsay (Paris, France). The synchrotron radiation source operated at an electron beam energy of 1.85 GeV with an average electron beam current of 250 mA. The X-ray beam had a cross section 0.5×2.0 mm at the sample level and 0.5×1.0 mm at the detector level. Indicating with $\boldsymbol{s}_0$ the characteristic vector of the incident beam (whose modulus is $1/\lambda$, where λ is the X-ray wavelength) and with $\boldsymbol{s}_1$ the characteristic vector of the scattered beam, the scattering vector $\boldsymbol{s}$ is defined $\boldsymbol{s} = \boldsymbol{s}_1 - \boldsymbol{s}_0$. The modulus of $\boldsymbol{s}$ is $s = (2\sin\theta)/\lambda$, where 2θ is the scattering angle. The experimental scattered intensity for each protein concentration was determined as a function of the s parameter in the range between 2.61×10^{-4} Å^{-1} and 5.7×10^{-2} Å^{-1}. Scattering data were collected at a fixed wavelength ($\lambda = 1.4878$ Å, $\Delta\lambda/\lambda = 10^{-3}$) on a position-sensitive proportional detector, positioned 1080 mm from the sample. The constant width for each channel, deduced from the experimental conditions, corresponds to an increment of the scattering parameters equal to $\delta s = 1.918$ Å^{-1}/channel. The counting time was 8×200 s. The background scattering arising from the buffered solvent as well as from the experimental setup (capillary, tube, air and slits) was recorded prior to analysis of each protein sample for the same acquisition times, and subtracted from the sample scattering curves. All the experiments were performed at room temperature. Trypsinogen and β-trypsin samples for SAXS experiments were prepared at three different concentrations (4, 7 and 10 mg/mL) for each solvent condition. In order 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 (Ascenzi et al. 1988). The pH profile was investigated between pH 3 and 9.2, in an approximate universal buffer [containing x mL solution (0.2 M in boric acid and 0.05 M in citric acid) and (200–x) mL of 0.1 M trisodium orthophosphate]. The NaCl effect was measured in 0.1 M sodium acetate buffer (pH 4).

Energy-dispersive X-ray diffraction experiments

The EDXD data were collected using an instrument developed in our laboratory (Caminiti et al. 1996). Briefly, incident polychromatic X-ray radiation is used and the diffracted beam is energy resolved by a solid-state detector located at a suitable scattering angle. The instrument, operating in vertical θ/θ geometry, is equipped with an X-ray generator (W target), a collimating system, step motors, and a solid-state detector connected via an electronic chain to a multichannel analyzer. A cell is placed at the common center of rotation of the diffractometer arms. The X-ray source is a standard Seifert tube operating at 45 kV and 35 mA whose Bremsstrahlung radiation is used. The fluorescence L lines of W are in the 8–11 keV range and do not interfere with the measurements being outside the region of interest. The detecting systems is composed of an EG&G liquid-nitrogen-cooled ultrapure Ge solid-state detector (ORTEC 92X) connected to a PC through ADCAM hardware. The collimating system consists of four adjustable W slits. Both the X-ray tube and the detector holding arms can rotate, with the aid of step motors, around their common center in order to reach the desired scattering angle (reproducibility $>0.001°$ in 2θ). A diffraction angle of 0.15° was selected to investigate the suitable position of the reciprocal space. Trypsinogen and β-trypsin were used at 7 mg/mL in the presence of 50 mM benzamidine. The pH profile and NaCl effects were determined under the same conditions chosen for the SAXS measurements.

Determination of catalytic parameters

The β-trypsin assay with Z-ArgONp was performed spectrophotometrically at 20 °C, measuring the spectral changes associated with the formation of free *p*-nitrophenol (Ascenzi et al. 1980). The spectral properties of *p*-nitrophenol released from Z-ArgONp are pH dependent (Antonini and Ascenzi 1980; Ascenzi et al. 1980) and therefore different extinction coefficients were used depending on the solution pH [at 360 nm and pH values between 4 and 6, ϵ (M, 1 cm) = 4500; at 405 nm and pH 8, ϵ (M, 1 cm) = 8900]. The absorbance increase on hydrolysis of Z-ArgNOp was monitored either in a Jasco V500 spectrophotometer or in an Applied Photophysics MV17 rapid-mixing stopped-flow apparatus. The initial velocity was corrected for the spontaneous hydrolysis of Z-ArgONp.

Structural analysis

Trypsinogen and β-trypsin (6 mg), both native and X-ray irradiated during EDXD experiments, were carboxymethylated with iodo[2-^{14}C]acetate as described previously (Barra et al. 1984). Aliquots (2 mg) of the carboxymethylated protein were suspended in 0.5 mL of 0.1 M ammonium bicarbonate (pH 7.5) and incubated at 37 °C after addition of 50 μg of trypsin for 3 h. The enzymatic digests were purified by using a Beckman Gold chromatography system on a macroporous reverse-phase column (Aquapore RP-300, 4.6 mm×250 mm, 7 μm, Brownlee Labs, USA) eluted with a linear gradient of 0–35% acetonitrile in 0.2% (v/v) trifluoroacetic acid, at a flow rate of 1 mL/min. Elution of the peptides was monitored using a diode array detector (Beckman model 168) at 220 nm and 280 nm. The amino acid sequence of the peptide samples was determined by automated Edman degradation using Applied Biosystem model 475A, 476A or 477A sequencers. Samples (0.2–1 nmol) were located onto poly(vinylidine difluoride) membranes (ProBlot, Applied Biosystem), coated with 2 μL polybrene (100 mg/mL, 50% methanol) and run with a Blott cartridge using an optimized gas-phase fast program. N-terminal sequence analysis of the protein was performed on samples electrotransferred on ProBlot membranes after SDS-PAGE (Matsudaira 1987) using a liquid-phase fast program.

Results

The intensity distribution scattered by a protein solution is a function of the modulus of the momentum trasfer s expressed as a function of the wavelength of the incident radiation λ and the scattering angle θ by the relation (Guinier and Fournet 1955):

$$s = (2\sin\theta)/\lambda \quad (1)$$

In SAXS experiments, λ is fixed while θ varies, whereas in EDXD experiments, θ is fixed while λ varies. In both cases the intensity distribution of the protein in solution can be approximated by the Guinier relation:

$$I(s) = I(0)\exp(-4\pi^2 s^2 R_g^2/3) \quad (2)$$

where $I(0)$ indicates the intensity at zero scattering angle and R_g is the radius of a spherical shell of the same mass and moment of inertia as the protein. Fitting of $\ln[I(s)/I(0)]$ versus s^2 permits the evaluation of the R_g value.

The Guinier plots of the data obtained with both SAXS and EDXD from bovine trypsinogen and β-trypsin are shown in Fig. 1. Under all experimental conditions, they give straight lines in the scattering region considered, a feature that indicates the monodispersity of the scatterers (i.e. particles) in solution; this means that no artifacts (such as molecular aggregates) are present in both systems, at least under the chosen experimental conditions. To provide more information on the molecular structure of both enzymes, SAXS data were plotted in Kratky graphs, i.e. $I(s)s^2$ versus s. This

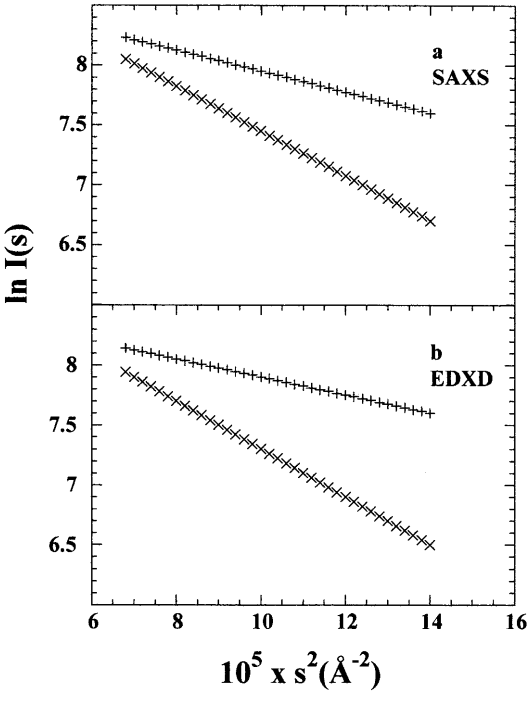

Fig. 1 Guinier plots of data collected by SAXS (**a**) and EDXD (**b**) at pH 6.2 for bovine β-trypsin (+) and trypsinogen (×). For details, see text

elaboration of the scattering profile enabled a direct characterization of the macromolecular compactness in that the presence of a peak observed in all graphs (Fig. 2) confirmed that, even under extreme conditions, both proteinases were globular in shape.

The pH profile of the R_g of β-trypsin, obtained by fitting the data collected at the LURE laboratory and those taken with EDXD, is shown in Fig. 3. It is evident that the experimental results with both techniques are in very good agreement. A maximum in R_g (=19.7 Å) is observed at pH 6.2; this value rapidly decreases on both abscissa sides until a constant value (18 Å at pH 5.4 on the left branch of the curve and 18.2 Å at pH 7 on the right branch) is reached. As expected, very similar data were collected for trypsinogen (Fig. 4), whose pH profile is simply shifted along the ordinate axis, the only difference being in a larger R_g value (by ~1 Å) relative to β-trypsin at all pH values. This evidence suggests that the smaller R_g value of β-trypsin relative to trypsinogen can easily be explained (Perkins et al. 1993) in terms of the removal of the extended amino-terminal hexapeptide of trypsinogen (see Introduction).

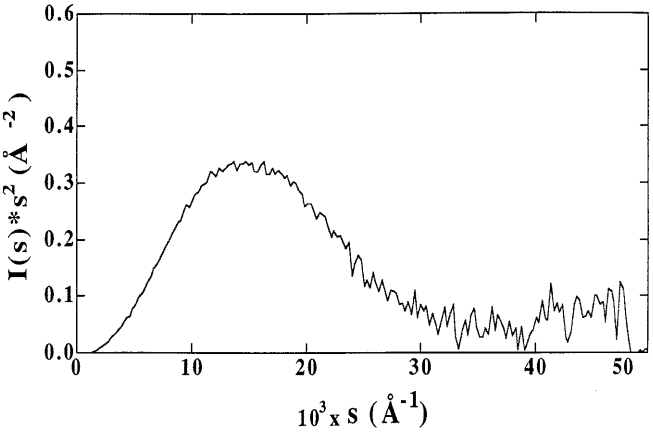

Fig. 2 Kratky plot of data collected with SAXS for bovine β-trypsin at pH 6.2 and 7 mg/mL protein. For details, see text

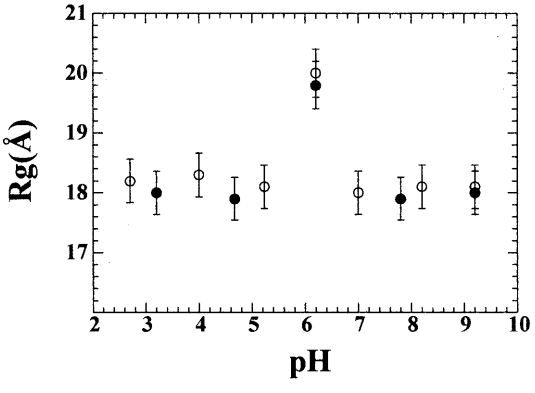

Fig. 3 pH profile of radius of gyration of bovine β-trypsin as measured by SAXS (●) and EDXD (○) in an approximate universal buffer. For details, see text

Since specific radiation damage in proteins has been reported (von Sonntag 1987; Weik et al. 2000), structural and functional investigations on the samples used for EDXD experiments were carried out in order to ascertain possible modifications of amino acid residues. Figure 5 shows the electrophoretic pattern of irradiated trypsinogen and β-trypsin under denaturing and nonreducing conditions in comparison with the corresponding native proteins. Under denaturing and either reducing or nonreducing conditions it is evident that no major damage (such as breakage of disulfide bonds) occurred during the acquisition of data by EDXD. Moreover, peptide maps of both proteinases after irradiation and in the native state, as well as their HPLC chromatograms (data not reported), gave identical

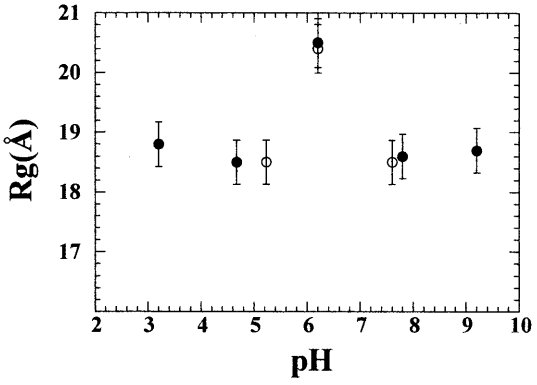

Fig. 4 pH profile of radius of gyration of bovine trypsinogen as measured by SAXS (●) and EDXD (○), in an approximate universal buffer. For details, see text

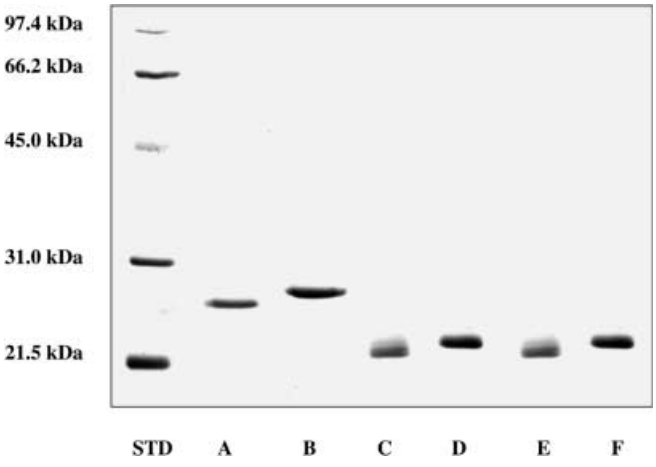

Fig. 5 Electrophoretic pattern of bovine β-trypsin and trypsinogen under denaturating (with sodium dodecyl sulfate) and reducing (with mercaptoethanol) or nonreducing conditions. *Lane STD*: protein standards with molecular masses; *lane A*: native β-trypsin (denaturated and reduced); *lane B*: native trypsinogen (denaturated and reduced); *lane C*: β-trypsin irradiated with X-rays during EDXD; *lane D*: trypsinogen irradiated with X-rays during data acquisition with EDXD; *lane E*: native β-trypsin (denaturated but not reduced); *lane F*: native trypsinogen (denaturated but not reduced)

results, forcing the conclusion that no structural damage was caused by EDXD radiation. These detailed findings at structural levels find support in the evidence (Fig. 6 and Table 1) that the catalytic properties of β-trypsin were not affected by the radiation flux in EDXD experiments. In addition, the observed values of steady-state parameters measured at three pH values (Table 1) are in fair agreement with those reported in the literature (Antonini and Ascenzi 1980), indicating that the structural stability of trypsinogen and β-trypsin during standard data collection of EDXD studies is independent of the protonation equilibria established by pH changes.

Previously reported R_g values for trypsinogen and β-trypsin, determined by X-ray and neutron scattering (Perkins et al. 1993), are less than, even though comparable with, the SAXS and EDXD data in Figs. 3 and 4. Since these differences are expected to depend on the solvent composition (Osterberg et al. 1980; Svergun et al. 1998), the effects on R_g of NaCl concentration at constant pH were determined. Thus, at pH 4 (Fig. 7) and 0.1 M acetate buffer the observed R_g value of β-trypsin ($\sim$17 Å) is in good agreement with that calculated for the hydrated structure of β-trypsin in cryogenic X-ray diffraction experiments (Nakasako 1999), but it is $\sim$1 Å less than that measured at the same pH and ionic strength (i.e. $R_g = 18.2$ Å) in the presence of other solvent components, i.e. borate, citrate and phosphate

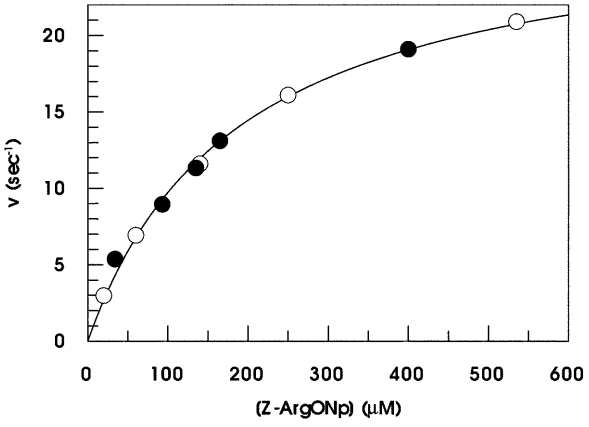

Fig. 6 Michaelis-Menten curve for esterolytic activity of bovine β-trypsin (10 μM) after irradiation with EDXD (○) and in native state (●) on *N*-benzyloxycarbonylarginine *p*-nitrophenyl ester (Z-ArgONp) at pH 7.0 and 20 °C

Table 1 Steady-state parameters (k_c and K_m) at 20 °C and different pH values for the hydrolysis of Z-ArgONp catalyzed by X-ray irradiated bovine β-trypsin (i.e. samples of β-trypsin after EDXD experiments). For additional information, see Materials and methods

pH	k_c (s^{-1})	K_m (μM)
4	6	300
6	28	188
8	136	84

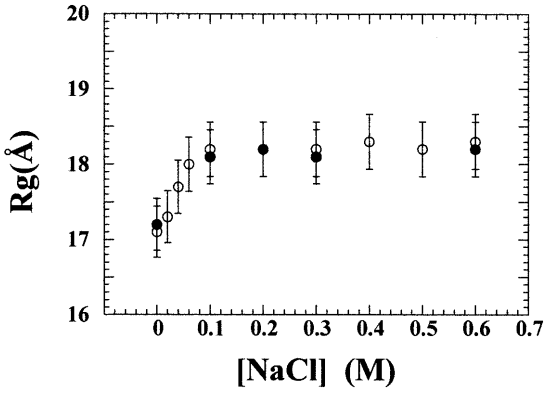

Fig. 7 Effect of NaCl on the radius of gyration of bovine β-trypsin as measured by EDXD (○) and SAXS (●)

(Fig. 3). Such evidence greatly supports the view (van Hippel and Schleich 1969) that strong electrolytes can affect the balance of interactions controlling protein conformation; in other words, the observed differences in R_g of β-trypsin at pH 4 (17 Å versus 18.2 Å) are expected to depend on different protein structure perturbants present in the two solutions (acetate versus borate, citrate, phosphate, the counterion being always the sodium ion). At constant pH (pH = 4), addition of NaCl was found to increase the β-trypsin R_g (see Fig. 6), which at high ionic strength tends to level off, suggesting that the observed modification in the overall protein geometry can depend on a direct binding of chloride to the macromolecule. In fact, when the proteinase shows a very high number of positively charged groups, i.e. in an acid environment, the dominant effect of salts is expected to be mainly due to anion binding; in addition, further evidence based on the sodium determination by a permselective membrane electode (Friedberg and Bose 1969) demonstrated that at pH 4 no sodium ion is bound to the serine proteinase α-chymotrypsin. On the other hand, in the case of trypsinogen the values of R_g actually go through a maximum (Fig. 8) that corresponds to a value of 20.5 Å at 0.07 M chloride. This bell-shaped curve is constituted by two branches with different

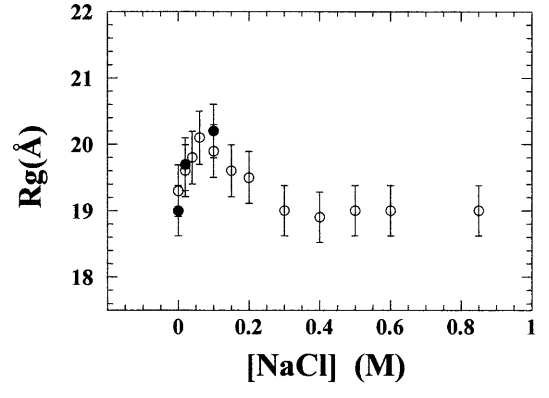

Fig. 8 Effect of NaCl on the radius of gyration of bovine trypsinogen as measured by EDXD (○) and SAXS (●)

slopes, suggesting the existence of at least two classes of binding sites with different affinity.

Discussion

As expected, EDXD and SAXS give the same information about overall geometric properties of dissolved particles, such as the R_g of trypsinogen and β-trypsin. It is relevant that specific or aspecific damages of these proteins were not observed after 12 h radiation, i.e. after the typical time for data collection with EDXD. This fact clearly depends on the low X-ray dose to which the protein samples were exposed as EDXD experiments proceeded, the overall dose being less than 10,000-fold that of a typical flux absorbed during data collection using moderate-intensity synchrotron radiation facilities (5×10^7 photons/s versus 5×10^{11} photons/s). The proteins used were among the best biological systems for investigating chemical and structural damages induced in macromolecules by X-rays. In fact, it is well known that disulfide bonds are particularly susceptible to X-ray irradiation (Weik et al. 2000) and both trypsinogen and β-trypsin contain in their molecule five disulfide bridges exposed to the solvent. The electrophoretic pattern (see Fig. 5) clearly indicates that, after irradiation with EDXD, no breakage of one or more of the disulfide bonds present in both proteinases was observed. Therefore, as a whole, the structural analysis of irradiated samples confirms that the EDXD technique is particularly suitable for studying protein systems.

The present investigation also provides experimental evidence for the specific binding of ions to trypsinogen and β-trypsin, in that the effects of solvent components on their R_g are all related to the particular nature of the salt rather than determined merely by the ionic strength of the environment as suggested by the Debye-Hückel theory. This conclusion is in line with crystallographic evidence. Thus, specific binding sites in proteases have been described for acetate (Johnson et al. 1999), phosphate (Edgcomb et al. 2000), and sulfate (Bartunik et al. 1989), even though in some cases different anions may compete for a single site (Johnson et al. 1999). These interactions appear to be at the basis of the observed variations of R_g in trypsinogen and β-trypsin. In fact, experimental evidence showed (Pleötil and Hlavatà 1988) that small-angle scattering from solutions of polyelectrolytes sensitively reflects the interaction between individual components in the (bio)chemical systems. In particular, the interaction of solvent molecules as well as of counterions with the charges on polyions (e.g. a protein) significantly contributes to the scattered intensity (Pleötil and Hlavatà 1988). Of course, such interactions can induce (depending on the magnitude of binding energy) local conformational changes or gross structural effects that propagate throughout the protein molecule, further influencing the intensity of the X-rays scattered at small angles. In serine proteases at pH 4, a minimum of two chloride ions are bound per macromolecule (Friedberg and Bose 1969). Therefore, in the simplest case it can be assumed that: (1) for β-trypsin, all chloride binding sites are equivalent and independent and, accordingly, as the slope of the curve shown in Fig. 7 approaches zero the binding sites are expected to become saturated with the anion; (2) for trypsinogen, at least two classes of chloride binding sites should be present in the macromolecule, classes that cannot be equivalent since both the mid-point of each transition (i.e. the first one from 0 to 0.07 M chloride and the other one from 0.07 to 0.3 M chloride) and the slopes of the two branches forming the whole curve are different (see Fig. 8). Therefore, since the two classes of groups capable of attaching chloride ions in trypsinogen must needs be topologically different, the conformational changes induced by saturation of low-affinity binding sites cannot be located at the same surface area as those produced by the action of anion association to the high affinity binding sites and therefore the modulation in R_g values is expected to depend on chloride concentration. Moreover, the high-affinity binding sites for chloride (i.e. those completely saturated at 0.07 M and producing an increase in R_g) appear to be present on both proteases, whereas the low-affinity chloride binding sites (i.e. those that reverse the phenomenon induced by the high-affinity binding sites) are exclusive for trypsinogen and possibly involve the activation domain of the zymogen.

The so-called pH effect, identical for both proteases, is characterized by a narrow peak in R_g values centered at pH$\approx$6.2. (see Figs. 3 and 4). Such a curve does not follow at all the activity profile as a function of pH typical of serine proteases acting on cationic substrates. In fact, the classical pH dependence of the various catalytic parameters of serine proteases shows (Ascenzi et al. 1980) a constant decrease in their values with decreasing pH from pH 8.5 to pH 5.5 (implicating an ionizing group with p$K\approx$7.1). This is not an unexpected result (Soman et al. 1989). Thus, even though changes in pH induce modifications of the surface net charge of both proteases, charged amino acids that are not part of the active site have no significant effect on the energetics of the catalytic mechanism [in that the low dielectric interior of the enzyme shields the active site by diverting the electric field through the high dielectric solvent (Soman et al. 1989)]. These considerations are in line with crystallographic results (Huber and Bode 1978) showing that free β-trypsin molecules at pH 5 and pH 8 are very similar in the overall three-dimensional structure. How then is it possible to interpret such an unexpected and unusual shape of the curves reported in Figs. 3 and 4? Whichever residue is protonated at low pH or negatively charged at high pH, its effect is felt outside the macromolecule, where not only protons (and hydroxide ions) but also three salts (sodium borate, potassium phosphate and sodium citrate) are present. Thus, a sort of cooperativity linking protons and anions is expected in these systems where simultaneous interacting equilibria are the rule. In other words, anions act by magnifying the standard pH effect on the R_g value,

that operates through (1) protonation and deprotonation of acid groups, and (2) formation and cleavage of salt bridges. From this viewpoint, the anion action can be understood as an increase in the pK of the groups involved in anion binding because of the negative charge of the anion itself; in other words, the pK shift introduced by anion binding results in an anion-induced proton uptake. Such an effect has been demonstrated in hemoglobin (Imai 1982). This model can account for the abnormally sharp branches forming the peak in Figs. 3 and 4, that suggest some cooperative proton binding. Therefore, the R_g values of the two proteinases as a function of pH can be visualized as the result of the intricate and cooperative interplay between the binding of protons, anions and water and the proteins. In particular, it can be assumed that the molecular events underlying the peak depend on the presence of two acid groups acting in opposite directions, just like the acid and alkaline Bohr effect in hemoglobin (Imai 1982). An additional consideration is in order. In the pH range where the change in R_g of both proteinases is observed (i.e. pH≈5.4 to pH≈7), only citrate (p$K_3 = 6.4$) and phosphate (p$K_2 = 7.2$) may efficiently contribute to this physical event (the pK of boric acid being > 9).

It is also interesting to notice that at pH 4 the overall effect on the trypsinogen R_g (∼19 Å) induced by the universal buffer (Fig. 8) matches that of acetate buffer (Fig. 4), whereas for β-trypsin (Fig. 7 versus Fig. 3) different R_g values (∼18 Å versus 17 Å) are measured under the two solvent conditions. These results are reminiscent of the different effects produced by (poly)anions on the compact and relaxed structures of hemoglobin (Amiconi et al. 1989; Coletta et al. 1999). Therefore, the above-mentioned findings relative to the two proteinases may tentatively be interpreted by assuming that the strains exerted by the various anions produce diverse structural effects on the two differently compact macromolecules. In particular, the interaction energy between anions and β-trypsin should easily propagate throughout this compact protein, thus determining modification of the overall shape (and, accordingly, changes in the R_g value); on the other hand, such energy should not spread far in trypsinogen, since it would be used for bringing about local rigidification of flexible domain(s) (Huber and Bode 1978) and therefore no gross conformational changes can be expected.

Lastly, since electrostatic effects are certain to play a central role in the future of macromolecular modeling (Warshel and Papazyan 1998), it can be concluded from the reported results that the solution properties (including the radius of gyration) of globular proteins will continue to play a major role in providing semi-quantitative pictures of biomedical systems.

Acknowledgements This work has been partially supported by funds from the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) from the National Research Council (CNR), and from the Italian Istituto Nazionale di Fisica della Materia (INFM).

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