Small-angle X-ray Scattering Study of Metal Ion-induced Conformational Changes in Serratia Protease*

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Metal ion-induced conformational changes in Serratia protease which contains one zinc ion per molecule were investigated by the small-angle x-ray scattering method. The molecule is an elongated ellipsoid of approximately 110 x 40 x 40 Å with a large cleft in its central region. Comparisons of the native (zinc-enzyme) with the zinc-free (apoenzyme) enzyme and with the zinc-replaced metalloenzyme show small but significant differences in their radii of gyration, maximum particle dimensions, and intraparticle pair-distance distributions. The radius of gyration and maximum particle dimension of the native enzyme are almost the same as those of the cobalt-enzyme but are shorter and longer, respectively, than those of the apo- and cadmium-enzymes. Simulation analysis based on the intraparticle pair-distribution function showed that these modified enzymes are comparable with the native enzyme in overall structure, and, except for the cobalt-enzyme, differ in cleft size. The residual enzymatic activity of the cobalt-enzyme is the same as that of the native enzyme, but the apo- and cadmium-enzymes have considerably less activity. The size of the cleft therefore is strictly controlled to ensure optimal enzyme activity, and the position and coordination behavior of the zinc ion in the cleft appears to be essential both for biological functioning and for the maintenance of the gross tertiary structure.

Serratia protease is an extracellular metalloprotease extracted from the culture media of Serratia sp. strain E-15 that contains one zinc ion per molecule. It is widely used in medicine as an anti-inflammatory agent and has been studied extensively by various physicochemical techniques. This enzyme is monomeric and consists of a single polypeptide chain of 470 amino acid residues with a molecular weight of 51,000. A comparison of its amino acid sequence indicates that the zinc chelating region is homologous to that of therapeutic enzymes. The reduction of the zinc ion from the native enzyme by EDTA and the zinc-replaced metalloenzyme by adding the corresponding metal ions as chlorides (CoCl2 and CdCl2) to the apoenzyme solution and dialyzing the whole, first against distilled water for 1 day, then against 0.01 M phosphate buffer, pH 7.0, for 3 days. Protein concentrations of the native and modified enzymes were determined spectrophotometrically by the UV absorption at 280 nm based on E1 cm=13.0 (7) (there is no difference in UV-spectra among these enzymes; Ref. 2). The activity of each enzyme was measured with casein as the substrate according to the procedure of Miyata et al. (7).

SAXS Measurement—The x-ray source was a 0.4 x 8-mm spot on the copper anode of a Phillips fine-focus x-ray tube operated at 40 kV, 36 mA with a Rigaku DRC' x-ray generator. Line focus geometry was used to obtain intense SAXS intensities. Scattered x-rays were recorded in the scattering range of 3.4 x 10−2 to 8.5 x 10−1 rad on a linear position-sensitive proportional counter (PSPC) at the sample-to-detector distance of 297.5 mm. The effective length of the counter (50 mm) was divided into 512 channels in the multi-channel analyzer, the primary x-ray beam being introduced near the center of the counter. Details of the construction of the SAXS camera and its data acquisition system are described elsewhere (8-10). Protein solutions and their buffer solutions, for background subtraction, were sucked into a thin-walled quartz capillary (inner diameter, 1.0 mm) by the injector through the polyethylene tube connected at the top of the capillary to attain the same transmission and/or path length between the protein and buffer solutions. The temperatures of the...

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The abbreviations used are: SAXS, small-angle x-ray scattering; PSPC, position-sensitive proportional counter; Rg, radius of gyration; Dmax, maximum particle dimension.
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sample solutions were strictly controlled at 15 ± 0.05 °C by circulating water in a constant temperature bath around the solutions. Seven successive measurements were made for each sample solution with an exposure time of 6000 s per measurement. The resulting seven data sets were totaled after inspections for x-ray radiation damage to the solution and the existence of instrumental artifacts.

Data Reduction—SAXS intensities measured at both sides of the primary beam were averaged at equivalent positions around the primary beam after correction for the positional sensitivity of the PSFC and subtraction of the background intensities. The position of the primary beam was refined so as to minimize the reliability factor, \( R_{\text{m}} \), of the SAXS intensities at the equivalent positions. Background intensities were measured from the outer buffer solutions used in the final dialysis. The slit-smearing effect was compensated for with the algorithm developed by Glatter (11). SAXS data for each protein solution were normalized to a unit protein concentration and used for the subsequent data analysis. The scattering parameter, \( q \), is defined by \( q = 4\pi\sin(\lambda)/\lambda \), where \( 2\theta \) is the scattering angle and \( \lambda \) the wavelength of x-rays used (\( \lambda = 1.5418 \AA \)).

Data Analysis—The SAXS intensities, \( I(q) \), from the protein solution were analyzed on the assumption that the solution was an ideal one in which identical globular particles were distributed randomly with no correlation to position and orientation. The SAXS intensity in such a case is the sum of the intensities scattered by individual particles.

The \( I(q) \) allows for the following close approximation in the innermost scattering region as shown in the following equation: \( I(q) = I(0) \cdot \exp(-R_e^2 q^2/3) \) (1)

\( R_e \) is the radius of gyration of the particle and \( I(0) \) the zero-angle scattering intensity. \( R_e \) and \( I(0) \) are derived from the Guinier plot \((\ln[I(q)] \text{ versus } q^2)\) (12), as the slope and intersection of the line least-squares fit in the \( q^2 = 0 \) range of \( 1.56 \times 10^{-3} \) to \( 5.00 \times 10^{-3} \) for all the protein solutions.

The intraparticle pair-distance distribution function, \( P(r) \), was calculated by Fourier transformation of \( I(q) \) (13) as shown in Equation 2.

\[ P(r) = \frac{1}{2\pi^2} \int I(q) - q^2 \cdot \sin(q \cdot r) \, dq \] (2)

\( P(r) \) is the probability of finding a given distance, \( r \), between any two volume elements of a particle, these volume elements being weighted with their respective excess electron densities (13). Because of finite resolution of the scattering parameter data, \( q \), the \( P(r) \) function was obtained from numerical integration from \( q = 0 \) to \( q = q_{\text{max}} \), the largest \( q \) value recorded on the PSFC. \( q_{\text{max}} \) is \( 0.34 \AA^{-1} \). The resulting termination effect and the counting errors in \( I(q) \), which cause artificial fluctuation of the function, were avoided by use of indirect Fourier transformation (14) and also of accurate intensity measurements up to the \( q_{\text{max}} \), at which value the scattering from the protein solutions was almost the same as from their buffer solutions, approaching the background levels. From the \( P(r) \) function, the maximum particle-dimension, \( D_{\text{max}} \), was estimated as the distance, \( r \), where the \( P(r) \) becomes zero.

The scattering volume, \( V \), that corresponds to the hydrate volume for soluble protein in solution was estimated from the following equation (15, 16).

\[ V = 2\pi^2 I(0)/Q \] (3)

\( Q \) is Porod’s invariant \((Q = \int I(q) \cdot q^2 \, dq)\). \( Q \) was obtained from the numerical integration of \( q = 0 \) to \( q = q_{\text{max}} \). No further integration beyond \( q_{\text{max}} \) was done, because the scatterings from the protein solutions reached background levels beyond that point.

To assess the interparticle interference effect that appears in smaller angle regions of \( I(q) \) data, we prepared protein solutions of three different concentrations (11, 8.7, and 6.0 mg/ml) for the native and modified enzymes. Careful inspection of the concentration dependence of the Porod parameter \( P(r) \) (11) showed that for each protein solution the effect was negligible within experimental error at concentrations of less than 8.7 mg/ml.

RESULTS AND DISCUSSION

Molecular Parameters and Intraparticle Pair-Distance Distribution—The Guinier plots of Serratia protease and its modified enzymes are shown in Fig. 1. They give straight lines in the given scattering regions which indicate monodispersity of the x-ray scatterers (particles) in solution; all the enzyme solutions consist of homologous particles, therefore, there are no artifacts such as particle aggregates. The molecular parameters for each enzyme are given in Table I. The \( R_e \) of the native enzyme differs significantly from those of the apo- and cadmium-enzymes. The differences in scattering volume, \( V \), are all within the experimental errors.

To provide more information on the molecular structures of the enzymes, we calculated their \( P(r) \) functions (Fig. 2). The profile of the native enzyme is similar to that of the cobalt-enzyme (Fig. 2b), but differs significantly from the profiles of the apo- and cadmium-enzymes (Fig. 2, a and c). The values near \( r = r_{\text{max}} \) (where \( P(r) \) has the largest value) of the native enzyme are larger than those for the apoenzyme and smaller than those for the cadmium-enzyme. By contrast, they are smaller than the values for the apoenzyme beyond \( r = 70 \AA \) and larger than those for the cadmium-enzyme beyond \( r = 60 \AA \). These results suggest that conformational change occurs during modification of the native enzyme.

Simulation Analysis for Model Building—As stated, the molecular parameters and \( P(r) \) functions of the native and modified enzymes are indicative of metal ion-induced conformational changes in Serratia protease. To show this, we built models of these enzymes. The conventional procedure for model building with SAXS is to calculate the theoretical \( I(q) \) functions of various models and compare them with the experimental \( I(q) \) data (simulation analysis in reciprocal space). We used the \( P(r) \) function instead of \( I(q) \) for our analysis (simulation analysis in real space) because simulation analysis in real space provides the direct information necessary to modify the structural parameters of the model examined. Analysis in reciprocal space, however, has problems with...
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FIG. 2. Pair-distance distribution functions, \( P(r) \) (thick line), of the apo- (a), cobalt- (b), and cadmium-enzymes (c). For comparison, the \( P(r) \) function of the Serratia protease (thin line) is superimposed on the respective functions of the modified enzymes.

The modification and often is hampered by its highly abstract nature in the innermost region of the \( I(q) \) data (13).

Model Building for Serratia Protease and Its Modified Enzymes—For the initial model in our simulation analysis, we used a simple triaxial body, an ellipsoid with a uniform electron density within it, because as low resolution structures most monomeric proteins closely approximate ellipsoids (Ref. 18; Ref. 19, and references therein). The initial model therefore was subjected to simulation analysis in real space on the assumption that the native and modified enzymes have ellipsoidal structures, i.e. the theoretical \( P(r) \) functions for ellipsoids of various triaxial lengths were calculated and compared with the experimental \( P(r) \) functions of the native and modified enzymes. All the ellipsoid models examined, however, gave poor agreement with the experimental functions. Their theoretical \( P(r) \) functions systematically are poorly fitted to the experimental functions both near \( r = r_{\text{max}} \) and beyond \( r = 50 \) Å. In these regions, the experimental functions respectively are significantly larger and smaller than those of the theoretical functions.

These systematic differences are indicative of the dimeric character of the Serratia protease structure because the \( P(r) \) functions of dimeric bodies with different subunit arrangements have in common a shoulder or, in some cases, subsidiary maximum beyond \( r = r_{\text{max}} \) which arises from the contribution of the pair-distance distribution between two subunits in the dimer (20). Although Serratia protease is monomeric and not a dimer, we carried out the model building on the basis of the concept that the enzyme has a well separated domain structure. In fact, it is generally accepted that large monomeric proteins often are composed of two or three compact domains and that their structures have a dimeric or trimeric character. The initial model therefore was modified to give it an ellipsoidal shape with domain structure.

This modified model is shown in the inset in Fig. 3. It was built from a conventional ellipsoid by dividing it equally along the plane perpendicular to its major axis then separating the resulting halves by an appropriate distance, \( x \), to produce the dimeric character. Using this model, we proposed various ellipsoid models that differed in \( x \) and the dimensions of the halves of the ellipsoid. Their \( P(r) \) functions were compared with the experimental function for the native enzyme. Unlike the analysis based on the conventional ellipsoid model, the \( P(r) \) function of the ellipsoid inset in Fig. 4a showed good agreement with the experimental function (Fig. 4a).

Before extending this analysis to the modified enzymes, we examined the profile dependency of the \( P(r) \) function on the distance, \( x \), which corresponds to the width of the cleft between the domains, using the best-simulated model for the native enzyme (Fig. 3). Results showed a systematic profile change in which an increase in cleft width produces a decrease in the distance distribution near \( r = r_{\text{max}} \) and an increase in distribution beyond \( r = 50 \) Å, indicating that the difference in the cleft size produces the significant difference in \( P(r) \) functions for the native and apoenzymes (Fig. 2a) and that for the native and cadmium-enzymes (Fig. 2c).

We therefore simulated the experimental \( P(r) \) functions of the modified enzymes for cleft width and, as expected, obtained the best-simulated models for the enzymes (Fig. 4, inset). Their theoretical \( P(r) \) functions are in good agreement with their experimental functions over the whole distance.

FIG. 3. Pair-distance distribution functions, \( P(r) \), of ellipsoid models with a cleft width, \( x \), of 0 Å (1), 6 Å (2), 8 Å (3), 10 Å (4), 12 Å (5), and 14 Å (6).
FIG. 4. Pair-distance distribution functions, $P(r)$, of the best-simulated models (thick line) for the (a) Serratia protease, (b) apo-, (c) cobalt-, and (d) cadmium-enzymes. The experimental $P(r)$ functions are superimposed as a thin line for comparison. The respective best-simulated models are drawn in the insets.

Table II

|        | N-EM | Apo-EM | Co-EM | Cd-EM |
|--------|------|--------|-------|-------|
| $x$ (Å) | 10   | 14     | 10    | 8     |
| Residual activity (%) | 100  | 0.5    | 100   | 20    |

range (Fig. 4). Incidentally, further simulation for the dimensions of the halves of the ellipsoids resulted in significant difference in $r_{max}$ values that causes a fairly poor agreement over the whole distance range. These results strongly indicate that the structural differences in the native and modified enzymes are only in cleft size, which view is partially supported by the fact that there are no distinguishable differences in the ORD curves and UV-spectra among these enzymes (2). The cleft widths of the best-simulated models for the native and modified enzymes are given in Table II. It follows that the native and modified enzymes are ellipsoidal molecules with overall dimensions of $110 \times 40 \times 40$ Å and consist of two almost identical domains separated by different distances. The structure of the native enzyme should change on removal of the zinc ion. And the structure of the resulting apoenzyme is reduced to that of the native enzyme upon introducing cobalt ion, whereas introduction of cadmium ion produces small but significant difference from the native enzyme structure.

Relationship between Cleft Size and Enzymatic Activity—Because zinc ion is essential for the activity of Serratia protease, we believe that the size of the cleft affects the enzyme's activity. The residual activities and cleft widths of the modified enzymes (Table II) show that their activities are highly dependent on cleft size. The cobalt-enzyme has the same activity as the native enzyme; whereas, the apo- and cadmium-enzymes have considerably less. This indicates that the active site of Serratia protease is near the cleft and that the cleft size of the native enzyme (10 Å) is crucial to the trapping of a substrate molecule. The cleft sizes of the apo- and cadmium-enzymes differ from the size of the native enzyme, thereby causing considerable loss of activity. The
The results of our study demonstrate that the SAXS method, which is based on accurate intensity measurements and careful data analysis, is a useful technique for the analysis of small conformational or structural changes in biological macromolecules in solution.

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