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The kinetics of conformational changes of α_2-macroglobulin determined by time resolved X-ray solution scattering

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Abstract

The rate of gross conformational change of α_2-macroglobulin (α_2M) during its proteinase trapping was directly determined for the first time using time-resolved X-ray solution scattering. Decrease of radius of gyration was observed under pseudo-first-order conditions with excess proteinases, which exhibited a monophasic timecourse. The rate constants were 0.5 ± 0.1 s^{-1} and 0.8 ± 0.2 s^{-1} for the reaction with chymotrypsin and trypsin, respectively. There was no concentration dependence of the observed rate constants. Therefore, the rate-limiting step of the gross conformational change was not the bimolecular encounter reaction between α_2M and proteinases, which requires a new proposal of pre-trapping of proteinases before the gross conformational change.

Key words: α_2-Macroglobulin; X-ray solution scattering; Time-resolved measurement; Trapping mechanism

1. Introduction

Human α_2-macroglobulin (α_2M) is a proteinase inhibitor of M_r 720,000 [1] present in plasma at a concentration of about 3 mg/ml. The protein is composed of four identical subunits linked in pairs by disulfide bonds and capable of inhibiting two trypsin or chymotrypsin molecules with one α_2M molecule. Unlike other proteinase inhibitors, α_2M does not block the active site of the proteinase but rather engulfs the whole enzyme molecule by the 'trap mechanism', as first proposed by Barrett and Starkey [2]. According to this proposal, a proteinase binds to a particular sequence in α_2M, named the 'bait region', and hydrolyzes a peptide bond therein. This causes a rapid conformational change that physically entraps the enzyme molecule within the α_2M molecule. Thus, the proposed entrapping conformational change is the key to the proteinase inhibitory action of α_2M.

Electron microscopy [3–7] and X-ray solution scattering [8,9] showed that a large conformational change occurs when α_2M traps proteinases. Such a conformational change was also detected by gel-electrophoresis [10], sedimentation analysis [11] and gel-chromatography [4]. Although fluorescence stopped-flow kinetic investigations were reported [12,13], the correspondence of the kinetics to the gross conformational change was not clear.

Parameters obtainable from X-ray solution scattering are limited but a few physically unambiguous parameters are obtainable, including the radius of gyration (R_g), and its feasibility in the study of time-dependent conformational change of bio-macromolecules is well established [14–19]. Moreover, as X-ray is scattered by electrons, a change of X-ray scattering directly represents the change of molecular shape. Here we report the rates of the gross conformational changes of α_2M induced by chymotrypsin and trypsin as determined by time-resolved X-ray solution scattering.

2. Materials and methods

2.1. Proteins

Human α_2M was purified from human plasma as described earlier [20] using polyethylene glycol precipitation, gel-chromatography and Zn^{2+} chelate chromatography. The final preparation was homogeneous by gel-electrophoretic criteria.

α-Chymotrypsin was purchased from Sigma Chemical Co. (St. Louis, MO). More than 95% of chymotrypsin molecules were active, as shown

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Abbreviations: α_2M, α_2-macroglobulin; R_g, radius of gyration, PMSF, phenylmethanesulfonyl fluoride.

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by active site titration with *p*-nitrophenyl acetate as substrate [21]. Inactive chymotrypsin was prepared by incubating chymotrypsin with 10 mM phenylmethanesulfonyl fluoride (PMSF) for 2 h at room temperature. Trypsin was purchased from Sigma Chemical Co. and the active molecule was purified through benzamidine-Sepharose (Pharmacia LKB, Uppsala, Sweden). α_2M–chymotrypsin complex and α_2M–trypsin complex were obtained by mixing α_2M with chymotrypsin or trypsin in the molar ratio 1:2 (α_2M:proteinase), followed by incubation at room temperature for 10 min.

2.2. X-ray solution scattering

X-ray solution scattering experiments were performed at the BL 15-A1 small-angle installation at the Photon Factory, Tsukuba, Japan. The mirror-monochromator camera gave an X-ray beam with a wavelength of 1.5 Å and cross-section of 2.9 mm (horizontal) × 2.0 mm (vertical) at the sample level. Data were recorded by a linear position-sensitive detector with 512 channels placed at 2.3 m from the sample [22]. The stopped-flow apparatus had two N_2 gas pressure-driven syringes, and a mixer designed for viscous solution. The mixed solution entered the X-ray scattering cell equipped with two quartz windows (50 μm thick), 1 mm apart from each other and with a 4 × 3 mm usable area. The mixing ratio (vol./vol.) was 1:1. The dead time of the device was estimated to be less than 10 ms [23]. The temperature of the apparatus was kept at 5°C with a Peltier cooling device.

The radius of gyration, R_g, was determined from the Guinier plot of scattering curve within the angular range 2.5×10^{-3} Å^{-1} < s < 7.5×10^{-3} Å^{-1}.

3. Results

Static X-ray small angle scattering patterns of α_2M before and after the reaction with chymotrypsin are shown in Fig. 1. When α_2M reacted with chymotrypsin, the scattering intensity increased in the small angle region and a side peak characteristic of intact α_2M disappeared. When α_2M reacted with trypsin a similar change of scattering pattern was observed, as reported by Österberg and Malmensten [9].

The Guinier plot of the scattering pattern of α_2M (Fig. 2) showed linearity for a fairly wide range in the small angle region and the linear region was more extended after the reaction with proteinases, as shown in Fig. 3. The radius of gyration (R_g), determined from the Guinier plot for 7.5 mg/ml α_2M, changed from 7.9 ± 0.5 nm to 6.8 ± 0.5 nm when α_2M reacted with chymotrypsin.

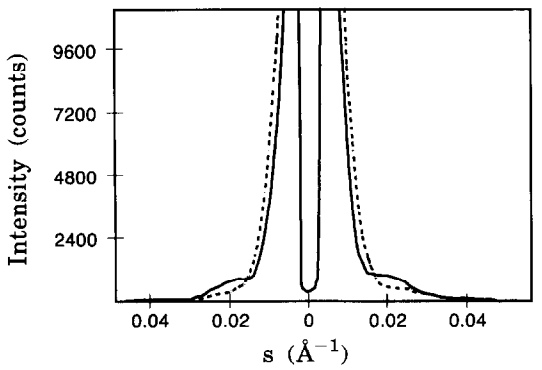

Fig. 1. X-ray solution scattering of α_2M. Small angle X-ray scattering curves for solutions of α_2M (7.5 mg/ml) before (———) and after reaction with chymotrypsin (- - -). Counts I (s) are plotted against s, where: s = $(2/\lambda)\sin\theta$, and 2θ is the scattering angle.

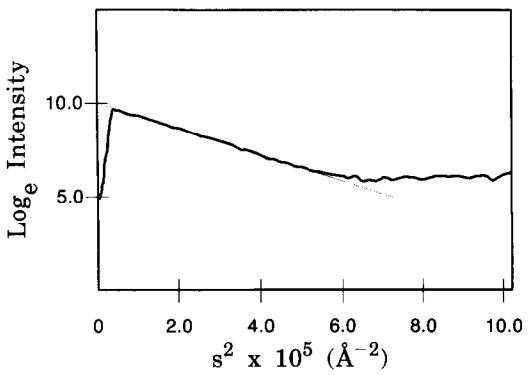

Fig. 2. Guinier plot of α_2M (7.5 mg/ml).

To observe the timecourse of the conformational change of α_2M induced by the reaction with proteinases, α_2M (typically 5 mg/ml) was mixed with an excess amount of chymotrypsin or trypsin in stopped-flow apparatus and X-ray solution scattering was measured. An example of calculated values of R_g^2 for the reaction mixture of α_2M and chymotrypsin were plotted against time in Fig. 4. The data showed a good fit to an exponential curve with a rate constant of 0.54 s^{-1}. The estimated R_g was 7.3 nm at time zero and 6.9 nm at the end point. When chymotrypsin was inactivated with PMSF and then mixed with α_2M, the observed R_g was constant at 7.3 nm (± 0.2 nm). Experiments were repeated with different concentrations of α_2M (10 mg/ml and 5.0 mg/ml at final) and the rate constant was confirmed to have no dependence on the concentration of α_2M. Fig. 5 is an example of calculated values of R_g^2 for the reaction mixture of α_2M and trypsin. This data was fitted with an exponential curve with a rate constant of 0.89 s^{-1}.

As summarized in Table 1, concentration dependencies of the rate constants were examined for chymotrypsin and trypsin to determine the order of the reaction. Although the concentrations of proteinases were changed by up to 10 times, no concentration dependency was observed. From this result, the rate-limiting step for the conformational change observed with X-ray solution

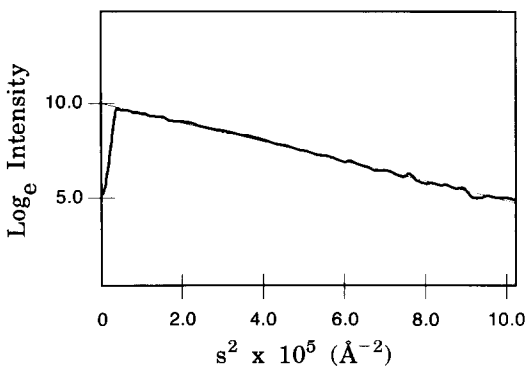

Fig. 3. Guinier plot of α_2M-chymotrypsin complex (7.5 mg/ml).

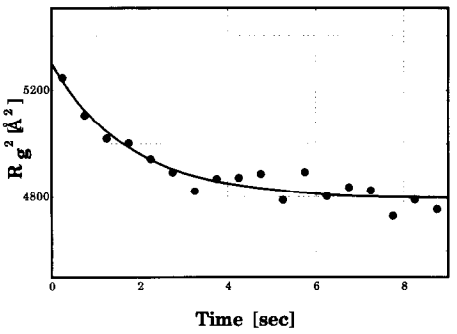

Fig. 4. Plot of the calculated R_g^2 of α_2M and chymotrypsin mixture as a function of time. α_2M (10 mg/ml) was mixed, in a ratio of 1.0:1.0 (vol.:vol.), with chymotrypsin (3.5 mg/ml). Both solutions were at 5.0°C in PBS. Each shot with a time-slice of 500 ms had a total volume of 180 μl, and 64 shots were accumulated. The solid curve is the fitting curve with a rate constant of 0.54 s^{-1}.

scattering was concluded to be the first order reaction for both chymotrypsin and trypsin.

4. Discussion

The observed R_g during the reaction was the apparent R_g of the mixture of macroglobulins before and after the transformation. The apparent R_g can be described as:

$$R_g^2 = \frac{(1 - x)M_a^2R_a^2 + xM_b^2R_b^2}{(1 - x)M_a^2 + xM_b^2}$$

where x is the molar ratio of the altered form of macroglobulin; M_a and R_a are the molecular weight and radius of gyration of macroglobulin before the conformational change; M_b and R_b are the molecular weight and radius of gyration of the altered form of macroglobulin [24]. As the molecular weight of chymotrypsin or trypsin is about 1/30 of that of macroglobulin, the difference of M_a and M_b is negligible. In this case the observed R_g can be expressed as:

$$R_g^2 = (1 - x)R_a^2 + xR_b^2 \qquad (M_a \approx M_b)$$

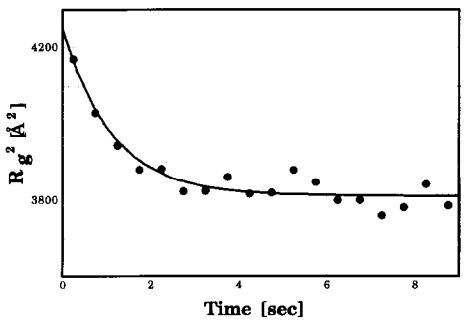

Fig. 5. Plot of the calculated R_g^2 of α_2M and trypsin mixture as a function of time. α_2M (10 mg/ml) was mixed, in a ratio of 1.0:1.0 (vol./vol.), with trypsin (3.2 mg/ml). Other experimental conditions were the same as in Fig. 4, and 66 shots were accumulated. The solid curve is the fitting curve with a rate constant of 0.89 s^{-1}.

Table 1
Rate constants of the gross conformational change

Proteinase	Concentration of proteinase (μM)		
	21	70	210
Chymotrypsin	0.50[a]	0.54[b]	0.44[b]
Trypsin	0.82[a]	0.89[b]	0.91[b]

Values are k_{app} (s^{-1}). Concentrations of α_2M were [a]2.0 μM, [b]7.0 μM.

This allowed us to analyze the observed timecourse of R_g^2 as an exponential curve and calculate the rate constant. Although the effect of free proteinase to the scattering profile was negligible, which we confirmed by measuring the scattering of the proteinase solution, the scatterings of proteinase were subtracted from all scatterings of solutions containing proteinase.

The conformational change detectable with X-ray solution scattering were actually observed within the time scale shown in Figs. 4 and 5. This was proved by the fact that the scattering patterns in the first frame of stopped-flow X-ray solution scattering were the same as those for intact macroglobulin, and the scattering patterns of the last frame of stoppedflow X-ray solution scattering were the same as those for macroglobulin reacted with proteinases. Further, when α_2M was mixed with inactivated chymotrypsin in the stoppedflow apparatus, the observed R_g value was consistent with the estimated value for time zero. It was also confirmed that no further change was observed in stoppedflow experiments when the full scales of time were expanded to 100 s.

The gross conformational change of α_2M during the trapping of proteinase was shown to follow a single exponential first-order reaction. This proved that the rate of conformational change was not limited by the encounter of proteinase but by one of the first-order reactions following the encounter, including the conformational change itself. This rate of the limiting step may correspond to k_2 proposed by Strickland et al. [13]. This means that the slower half of the TNS (6-(*p*-toluidino)-2-naphthalenesulfonic acid) fluorescence increase had corresponded to the gross conformational change.

The rate constants for chymotrypsin and trypsin seemed to be different, which suggests the existence of a reaction step that is not rate limited by the proteinase encounter but is still proteinase dependent. This proteinase-dependent rate-limiting step may or may not be the cleavage of the bait region. Recently we measured the rate of bait region cleavage using SDS-PAGE [25] and pH-indicating dye (in preparation), which showed the existence of cleavage that was faster than the rate shown here. These results suggest that the cleavage of a second bait-region or an unknown proteinase-dependent reaction following bait region cleavage is the rate-limiting step for the conformational change rather than the first bait region cleavage.

The cleavage of a peptide bond by serine proteinase occurs in two steps: first is the acylation of the enzyme and second is the deacylation of the enzyme. As proteinase leaves the carboxyl-terminal of the cleaved peptide bond at the deacylation step, α_2M has to trap proteinase before deacylation, which was estimated to be around 30 s^{-1} for the first bait region cleavage from the result mentioned above. The observed conformational change seems to be much slower than the first bait region cleavage, although the proteinase that cleaves the bait region rarely escapes from α_2M. The gross conformational change may not be the trapping action but rather may be the appearance of a recognition site for e.g. a receptor. Otherwise there may exist some mechanism to prohibit the escape of proteinase resulting in the cleavage of the second bait region, thus gaining time for α_2M to undergo the gross conformational change. More experiments, including direct measurement of deacylation, are necessary to come to a conclusion about this.

We hope our result gives a further clue to the understanding of the mechanism of molecular trapping which is one of the most intelligent systems of a single molecule.

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