Trypsin complexed with α_1-proteinase inhibitor has an increased structural flexibility

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Abstract **Mutant rat trypsin Asp[189]Ser was prepared and complexed with highly purified human α_1-proteinase inhibitor. The complex formed was purified to homogeneity and studied by N-terminal amino acid sequence analysis and limited proteolysis with bovine trypsin. As compared to uncomplexed mutant trypsin, the mutant enzyme complexed with α_1-proteinase inhibitor showed a highly increased susceptibility to enzymatic digestion. The peptide bond selectively attacked by bovine trypsin was identified as the Arg[117]–Val[118] one of trypsin. The structural and mechanistic relevance of this observation to serine proteinase-substrate and serine proteinase-serpin reactions are discussed.**

Key words: α_1-Antitrypsin; Mutant rat trypsin; Acyl enzyme intermediate; Limited proteolysis; Induced fit

1. Introduction

According to Pauling's transition state theory [1], enzymes provide templates which are complementary to the reactants in their activated transition state rather than to the substrates in their ground state. Despite 50-year extensive research along these lines the molecular mechanism by which enzymes convert substrates from the ground state to the transition state is not clear at all. It is generally accepted, however, that the structure of the enzyme must undergo some changes to complement changes of the substrate throughout catalytic reactions [2–4]. The extent of these changes may depend on the enzyme-substrate system studied, varying from an extensive distortion of the enzyme structure to complement transition state (induced fit) to relatively minor, local conformational changes in the course of catalysis [2–4]. In the case of serine proteinases, the latter mechanism seems to be likely, since the structures of crystalline trypsin and chymotrypsin are undistinguishable from those of the same enzymes complexed with 'substrate-like' canonical proteinase inhibitors [3–5]. The question, however, is whether the binding of these inhibitors does properly mimic substrate binding. Dufton [6] argued that the modes of interaction of inhibitors and substrates with the same serine proteinase might be markedly different, and that binding of true substrates to the proteinase, unlike that of inhibitors, might induce a relative movement of the two large domains in the enzyme.

According to this hypothesis, such movement would focus stress on the scissile bond in the substrate, thereby facilitating its hydrolysis [6]. Our own enzyme kinetic studies on trypsin, chymotrypsin and mutant trypsins with modified substrate-binding pockets further support the view that serine proteinase action may be accompanied by some conformational changes of the enzyme [7,8]. Our interpretation of the poor catalytic activity of mutant trypsins with chymotrypsin-like substrate-binding pockets has been that though the pockets properly accommodate the substrate, this interaction does not induce the conformational change crucial for optimal transition state stabilisation [7–10]. Furthermore, we hypothesised that different conformational flexibilities of the substrate-binding sites of trypsin and chymotrypsin, rather than evident differences between the crystalline structures of the binding sites, represent the structural basis for the different substrate specificities of these proteinases [9,10]. Recently, this possibility has also been entertained by Perona and co-workers [11] when interpreting crystallographic data on two mutant trypsins with chymotrypsin-like specificity.

To directly explore the molecular mechanism of serine proteinase action time-resolved diffraction studies will be required. Until such studies will be technically feasible to be performed structural investigation of any kinetically stable reaction intermediates of serine proteinases may provide us with useful information about the extent and nature of conformational changes that the enzymes may undergo throughout catalysis. In this paper we present data on a dramatic conformational change of mutant trypsin Asp[189]Ser upon its interaction with α_1-proteinase inhibitor.

2. Materials and methods

2.1. Enzymes, inhibitors and chemicals

Bovine pancreatic trypsin TPCK and porcine pancreatic elastase were purchased from Sigma. Mutant rat trypsinogens, Asp[189]Ser and His[57]Ala, Asp[102]Asn, were expressed in an *E. coli* expression-secretion system, purified to homogeneity and activated by enterokinase, as described previously [7,12]. Human α_1-PI was obtained from Serva and purified to homogeneity on a MONO Q column (Pharmacia FPLC system, Uppsala, Sweden) by using a linear gradient from 0 to 0.5 M NaCl in 10 mM sodium phosphate buffer, pH 7.0. Fractions were monitored by SDS-PAGE [13]. The purified inhibitor was stored at −20°C. All chemicals used were of reagent grade.

2.2. Polyacrylamide gel electrophoresis and protein content determination

SDS-PAGE was carried out in 12% or 15% (w/v) slab gels according to Laemmli [13]. Native non-denaturing PAGE was performed under the same conditions except that SDS and 2-mercaptoethanol were absent from the buffers and that the polyacrylamide gel concentration was 10% (w/v). Protein content was determined by the method of Bradford [14].

2.3. Preparation and isolation of the mutant trypsin–α_1-PI complex

α_1-PI in a slight molar excess was mixed with mutant trypsin Asp[189]Ser in 10 mM sodium phosphate buffer, at pH 7.0. The mixture was loaded onto a MONO Q column and eluted with a linear pH and salt gradient from 7.0 to pH 5.0 and from 0 to 0.2 M NaCl in 10 mM sodium citrate-phosphate buffer. The separation was followed by SDS-PAGE [13]. The purified complex was stored at $-20°$C.

2.4. Limited proteolysis

Mutant trypsin Asp[189]Ser and its complex with α_1-PI, in a final concentration of 4 μM, in 0.2 mM sodium phosphate buffer of pH 7.0 containing 0.1 M NaCl were incubated with a 5 to 1 molar ratio of bovine trypsin. The digestion was performed at room temperature for different time periods from 0 to 16 h. Aliquots taken were boiled for 3 min and then PMSF was added to them at a final concentration of 1 mM. The samples were analysed by SDS-PAGE applying 15% and 12% (w/v) polyacrylamide gels for the mutant trypsin and the complex, respectively, and by N-terminal amino acid sequence analysis.

2.5. Separation by HPLC

Reversed-phase HPLC was used for the separation of fragments in the intact and enzymatically degraded complexes. Samples were loaded onto an Aquapore OD300 C18 column (Applied Biosystems, 4.6 mm × 220 mm) equilibrated with water containing 0.1% (v/v) TFA (solvent A), and a 30 min linear gradient was applied by using a mixture of 80% (v/v) acetonitrile, 20% (v/v) water and 0.08% (v/v) TFA, as solvent B. The flow rate was 1 ml/min and detection was done at 220 nm.

2.6. N-terminal amino acid sequence and amino acid analyses

N-terminal amino acid sequences were determined in an Applied Biosystems 471 A pulsed liquid-phase sequencer by using a program adopted from Hunkapiller and co-workers [15]. Amino acid analysis was performed by Pico-Tag method [16].

3. Results

Human α_1-proteinase inhibitor and mutant rat trypsin Asp[189]Ser were mixed and the mixture was subjected to chromatography on a MONO Q column as described in section 2. 85% of the protein content of the mixture was eluted in one peak and found to be homogeneous by both SDS-PAGE and native non-denaturing PAGE (Figs. 1 and 2). The molecular weight of the mutant trypsin Asp[189]Ser–α_1-PI complex as estimated by SDS-PAGE (Fig. 1) is about 73 kDa, somewhat less than the sum of those of trypsin (24 kDa) and α_1-PI (53 kDa). Parallel experiments with wild-type rat trypsin and α_1-PI resulted in similar results, except that the complex isolated from
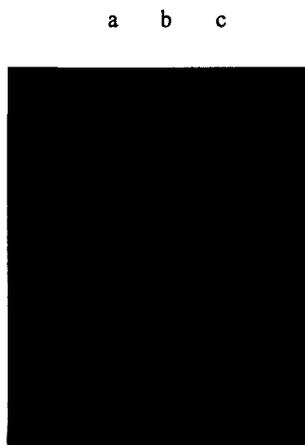

Fig. 1. 12% SDS PAGE of (a) trypsin mutant Asp[189]Ser, (b) α_1-PI and (c) purified trypsin mutant Asp[189]Ser–α_1-PI complex.

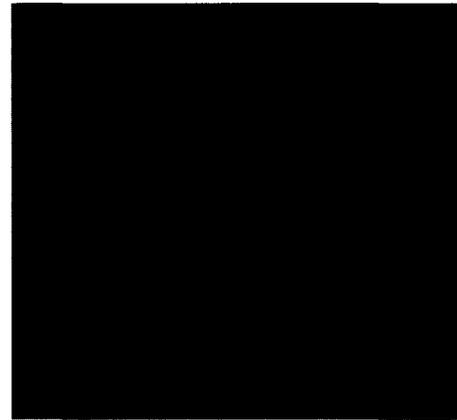Fig. 2. 10% native PAGE of trypsin mutants (a) Asp[189]Ser and (b) His[57]Ala, Asp[102]Asn, (c) α_1-PI, the equimolar mixtures of α_1-PI with (d) trypsin mutant Asp[189]Ser, and (e) trypsin mutant His[57]Ala, Asp[102]Asn.

their mixture was less homogeneous and stable than that of mutant trypsin Asp[189]Ser–α_1-PI (data not shown).

A rat trypsin mutant with destroyed catalytic triad, mutant His[57]Ala, Asp[102]Asn [12] was not able to form stable complex with α_1-PI as shown by native PAGE (Fig. 2).

To compare the structural flexibilities of mutant trypsin Asp[189]Ser in complex with α_1-PI and in its uncomplexed form, both preparations were digested with bovine trypsin. SDS-PAGE was used to follow the time-course of the digestion (Fig. 3). As seen in the Figure, the complex is much more susceptible to proteolysis than the trypsin mutant is in its uncomplexed form. In fact, the protein band of mutant trypsin–α_1-PI complex was converted to a new electrophoretic component within 15 min, while mutant trypsin showed resistance to tryptic digestion. It has to be noted that the presence of the cleaved form of α_1-PI appeared in these gels as a consequence of the 3 min boiling of samples.

Sequences of the first six N-terminal residues of α_1-PI, trypsin mutant Asp[189]Ser, the isolated mutant trypsin–α_1-PI complex and a 1-h tryptic hydrolysate of the latter one were determined, and the results are shown in Table 1. The N-terminus of α_1-PI is probably blocked since no amino acids could be detected in the first six cycles of Edman degradation. Sequencing the isolated complex, in addition to the N-terminal sequence of the mutant trypsin a new amino acid sequence appeared that corresponds to residues 359–364 of α_1-PI. PTH-amino acid signals for the two sequences were found to be comparable in each cycle indicating that α_1-PI was completely cleaved at peptide bond Met[358]–Ser[359]. The separation and identification of the C-terminal fragment, residues 359–394, of α_1-PI (Peak I in Fig. 4A, Table 1) by reversed-phase chromatography [17] of the mutant trypsin–α_1-PI complex confirmed this finding. N-terminal sequence analysis of a 1-h tryptic digest of the trypsin mutant–α_1-PI complex revealed the presence, in equimolar amounts, of three N-terminal sequences (Table 1). The new N-terminal sequence released by limited tryptic hydrolysis of the complex is Val-Ala-Thr-Val-Ala-Leu-, representing the 118–123 sequence region of rat trypsin. When the 1-h tryptic digest of the mutant trypsin–α_1-PI complex was subjected to
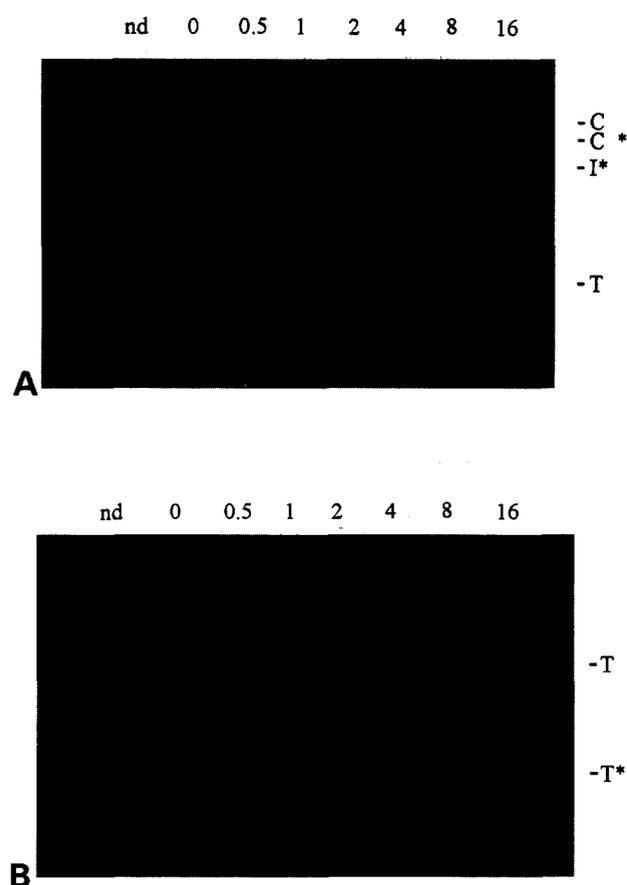
nd   0   0.5   1   2   4   8   16

-C
-C *
-I*

-T

A

nd   0   0.5   1   2   4   8   16

-T

-T*

B

Fig. 3. Limited proteolysis with bovine trypsin of rat trypsin mutant Asp[189]Ser–α_1-PI complex (12% SDS-PAGE; panel A) and of rat trypsin mutant Asp[189]Ser (15% SDS-PAGE; panel B). 4 μM complex and 4 μM mutant trypsin were incubated with 0.8 μM bovine trypsin at pH 7 (0.1 M NaCl, 0.2 M sodium phosphate buffer), at room temperature for different time periods (0–16 h). Aliquots removed at the indicated times (h) were boiled for 3 min and then PMSF was added at 1 mM final concentration to inactivate trypsin. nd (not digested) is the control sample which was incubated for 16 h under similar conditions but without bovine trypsin. T: trypsin mutant Asp[189]Ser (Mw 24 kDa); T*: C-terminal fragment of cleaved trypsin mutant (Mw 13 kDa); C: trypsin mutant Asp[189]Ser–α_1-PI complex without the C-terminal fragment of α_1-PI (Mw 73 kDa); C*: digested complex (Mw 62 kDa); I*: cleaved α_1-PI (without its C-terminal fragment; Mw 49 kDa).

reversed-phase HPLC, the newly released N-terminal fragment of the trypsin mutant coeluted with the C-terminal fragment of α_1-PI (Peak I in Fig. 4B, Table 1)

Schematic representation of the structures of trypsin mutant–α_1-PI complex and its trypsin-nicked form is based on the protein analytical work described above (Fig. 5).

4. Discussion

The exact molecular mechanism by which serpins including α_1-proteinase inhibitor interact with serine proteinases has not yet been established [18,19]. Since the reactive sites of serpins are susceptible to their target proteinases just like those of true substrates and 'substrate-like' canonical proteinase inhibitors, the general kinetic mechanism of their reaction with the enzymes can be written as follows:

$$E + I \Leftrightarrow E \cdot I \Leftrightarrow E \equiv I \rightarrow E - I^* \rightarrow E + I^*$$

Scheme 1

where E·I is the initially formed Michaelis complex, E ≡ I is the tetrahedral complex (TI) and E – I* is the acyl-enzyme intermediate that dissociates into the active enzyme (E) and the cleaved inhibitor (I*). The inhibitory mechanism of serpins differs, however, in many respects from that of canonical 'standard mechanism' inhibitors like kunins and kazals [18,19]. One striking difference is that the structure of serpins unlike that of canonical inhibitors, while reacting with the proteinases, undergo a dramatic change from a stressed, labile conformation to a relatively ordered, heat-stable form [18,19,20]. Furthermore, serpins unlike canonical proteinase inhibitors form kinetically stable complexes with proteinases [21,22]. The question if the general structure of such complexes corresponds to a tetrahedral [22] or an acyl-enzyme intermediate [19,21] is still debated.

Our own sequencing data on the mutant trypsin Asp[189]Ser–α_1-PI complex showed that the P_1-P_1' peptide bond was present in a cleaved form (Table 1, Fig. 4), thus supporting the view that the acyl-enzyme intermediate rather than the tetrahedral one may be the kinetically stable form of the complex. There may be some faint doubt, however, that the acyl intermediate was formed from the tetrahedral one under the conditions of phenyl-thiocarbamylation of the protein (first reaction of the Edman-degradation). Model experiments to exclude or confirm this possibility are now being performed in our laboratory. Our failure to form a stable complex between α_1-PI and mutant trypsin His[57]Ala, Asp[102]Asn further confirms the general notion that the catalytic apparatus of the proteinase is essential for complex formation with serpins [18,19,21,22].

Instead of native trypsin, a mutant trypsin with a single amino acid replacement in its substrate-binding pocket, Asp[189] to Ser [7], was used in this work. The low (auto)catalytic activity, 5 orders of magnitude smaller than that of wild-type trypsin, made this mutant an apparently ideal model to study the biochemical properties of the enzyme–α_1-PI complex. Since the X-ray structure of mutant rat trypsin Asp[189]Ser complexed with

Table 1
Results of the N-terminal amino acid sequence analysis of (1) α_1-PI, (2) trypsin mutant Asp[189]Ser, (3) trypsin mutant Asp[189]Ser–α_1-PI complex, (4) complex digested with bovine trypsin for 1 h and (5) HPLC peaks in Fig. 4 (for details see text)

Sample	No. of cycles					
	1	2	3	4	5	6
(1) α_1-PI	–	–	–	–	–	–
(2) Trypsin mutant	I	V	G	G	Y	T
(3) Complex	I	V	G	G	Y	T
	S	I	P	P	E	V
(4) Digested complex	I	V	G	G	Y	T
	S	I	P	P	E	V
	V	A	T	V	A	L
(5) HPLC peaks:						
peak I/Fig. 4A	S	I	P	P	E	V
peak II/Fig. 4A	I	V	G	G	Y	T
peak I/Fig. 4B	I	V	G	G	Y	T
	S	I	P	P	E	V
peak II/Fig. 4B	V	A	T	V	A	L

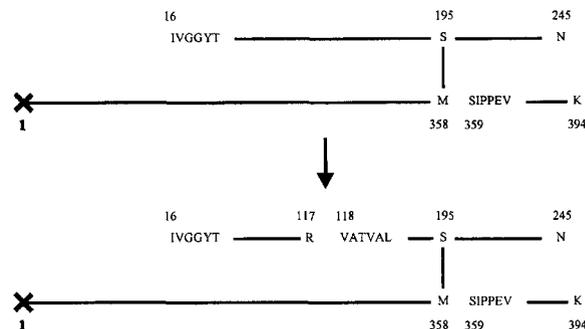The amino acids identified in the first six cycles are shown.
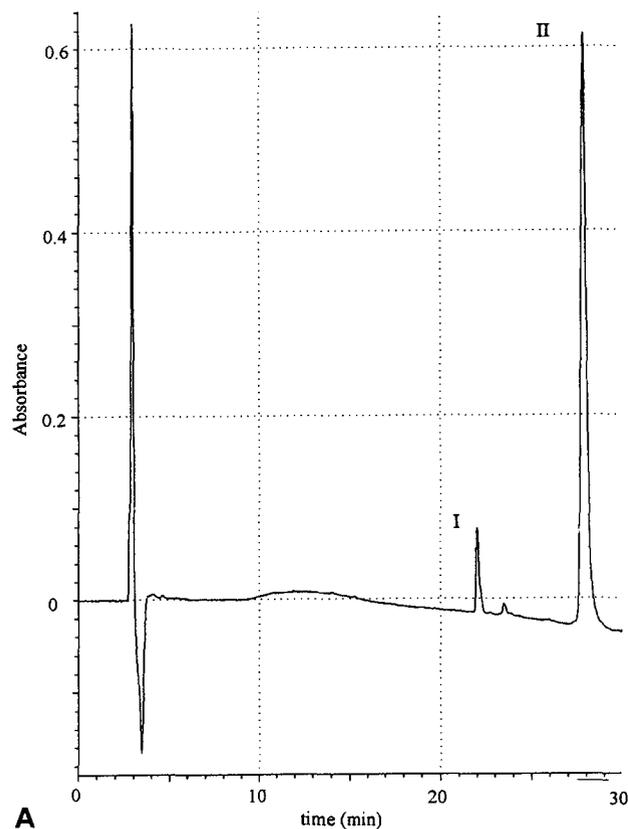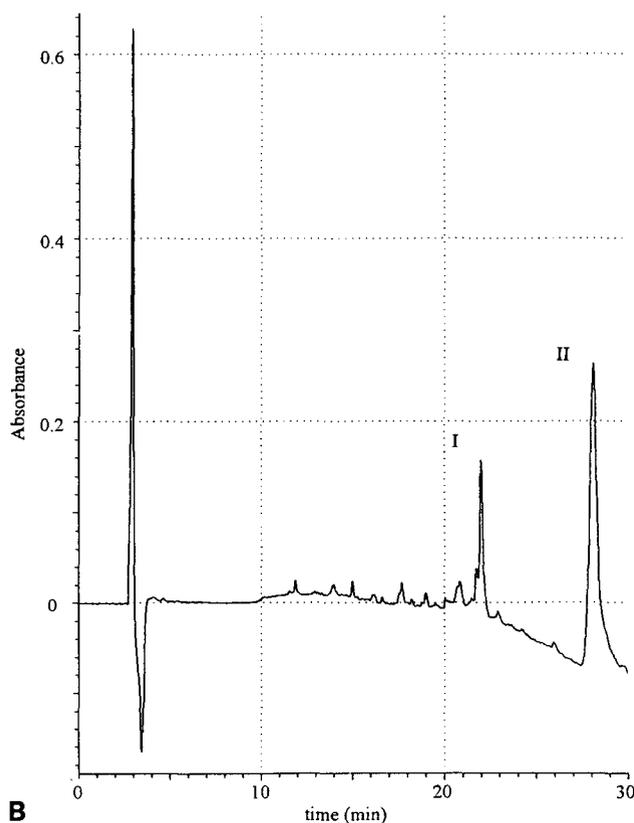
Fig. 5. Schematic representation of the structures of mutant trypsin-α_1-PI and its trypsin-nicked form.

bovine pancreatic trypsin inhibitor was shown to be identical with that of bovine trypsin complexed with the same inhibitor [23] there is no reason to doubt that structural features of the mutant trypsin Asp[189]Ser-α_1-PI complex are relevant to naturally formed proteinase-serpin complexes.

Though a relatively increased sensitivity of different proteinase–serpin complexes to proteolytic attack has already been noted in the literature [24,25], the cleavage sites within the complexes have not yet been identified. For the first time, our present studies provide evidence that the proteinase component of a proteinase–serpin complex underwent a dramatic conformational change upon complex formation with the serpin, and that this increased molecular flexibility of the enzyme serves as the structural basis for the marked susceptibility of the complex to proteolytic attack. The tryptic cleavage site identified as peptide bond Arg[117]–Val[118] in trypsin is of particular interest. This site is located within the hinge region connecting the two large domains of trypsin, the relative movement of which was postulated to play a crucial role in the catalytic process [6]. Considering the rational possibility that our mutant trypsin-α_1-PI represents either the acyl-enzyme or the tetrahedral intermediate of the proteinase–serpin reaction we would be tempted to propose that the proteinase might undergo a similar conformational transition when reacting with its true substrate. However, other possibilities have to be also entertained. Such possibilities are that only serpins can induce such a conformational distortion of target proteinases or that they just reinforce catalytically relevant structural changes of proteinases. Such changes of the enzyme structure would complement, at certain

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Fig. 4. Reversed-phase HPLC analysis of (A) intact complex, (B) complex digested with bovine trypsin for 1 h, injected under reducing conditions (2% v/v 2-mercaptoethanol). HPLC analysis was performed on a C_{18} reversed-phase column using a linear gradient of 0–80% acetonitrile in 0.1% (v/v) aqueous TFA over 30 min at a flow rate of 1 ml/min. Peaks were identified by N-terminal amino acid sequence and amino acid analyses (Table 1). Peak I in (A) is the C-terminal fragment of α_1-PI, (Ser[359]-Lys[394]); peak II in (A) is the trypsin mutant Asp[189]Ser-α_1-PI complex without the C-terminal fragment of α_1-PI; peak I in (B) contains the C-terminal fragment of α_1-PI (Ser[359]–Lys[394]) and the N-terminal fragment of trypsin mutant Asp[189]Ser (Ile[16]–Arg[117]); peak II in (B) is the trypsin mutant Asp[189]Ser-α_1-PI complex without the N-terminal fragment of trypsin (Ile[16]–Arg[117]) and the C-terminal fragment of α_1-PI (Ser[359]–Lys[394]).

stages of the proteinase–serpin interaction, the remarkable structural transition of serpins from a stressed conformation to a relaxed one.

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