Rabbit α -1-antiproteinase E: a novel recombinant serpin which does not inhibit proteinases

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A cDNA coding for the E isoform of α -1-antiproteinase (also called α -1-antitrypsin or α -1-proteinase inhibitor) was isolated by oligonucleotide hybridization following immunochemical screening of the rabbit liver cDNA library. The deduced amino acid sequence of the E isoform showed 96.4% identity in 413 residues of the F and S-1 isoforms of rabbit α -1-antiproteinase. The N-terminal half of the amino acid residues of the three isoforms was almost identical, but the putative reactive-site loop structure (P8–P'8) was significantly different in the various forms, the P1 site of the E form being glutamic acid. Interaction of the recombinant E form with the various proteinases was investigated by SDS/PAGE, followed by immunoblot analysis. The recombinant protein and trypsin formed a 62 kDa equimolar complex, which gradually became graded to the 37 kDa fragment through several intermediates. The E form also formed a complex of a

similar size with elastase and became degraded to the 31 kDa fragment. Several proteinases which cleaved the E form without forming a detectable complex on SDS/PAGE are chymotrypsin, protease V8, pancreas kallikrein, thermolysin, papain and ficin. Other proteinases, with a stringent substrate specificity, such as thrombin, factor Xa, plasmin, plasma kallikrein and cathepsin G, did not attack the E form. Unlike the F and S-1 forms of rabbit plasma α -1-antiproteinase, the recombinant E form did not inhibit the amidolytic and proteolytic activities of trypsin. Neither elastase nor protease V8 was inhibited by the E form. Thus the change in the amino acid residues in the reactive-site loop, probably in the P1 site, is responsible for the loss of inhibitory activity of rabbit α -1-antiproteinase E. The novel character of the E form could provide a new insight into the interaction of serpin and proteinases.

INTRODUCTION

Mammalian plasma contains several serine proteinase inhibitors, referred to as serpin [1], and α -1-antiproteinase (AP), also called α -1-antitrypsin or α -1-proteinase inhibitor, is present in the greatest concentration and accounts for most of the plasma trypsin-inhibitory capacity [2]. Human AP, which is capable of inhibiting a variety of serine proteinases, including trypsin, chymotrypsin and elastase [3,4], has been most extensively investigated with respect to its protein and gene structures [5,6], regulation of its biosynthesis, various mutations in relation to the development of pulmonary emphysema and other diseases [4,7], but the mode of interaction between the inhibitor and the proteinases has been little clarified. Three-dimensional structures of the uncleaved form of antithrombin III and an α -1-antichymotrypsin variant have recently been determined [8–10], and it was confirmed that the intact reactive-site loop of inhibitory serpins contains a distorted helical conformation, from which the structure of intact AP could be inferred. In order to reveal the reaction mechanism by which the proteinase is inhibited, a study of the interaction of various APs with their target proteinases would be fruitful [11,12].

Two forms of AP, designated as F and S according to the mobility in non-denaturing PAGE, were isolated from rabbit plasma [13]. The F form, which is the major isoform in the plasma, inhibited trypsin, chymotrypsin and elastase in a stoichiometric manner [12,13]. On the other hand, the S form did not inhibit trypsin, but protected it from being inactivated by soybean

trypsin inhibitor, resulting in trypsin-protein amidase activity [12,14], a characteristic of α -macroglobulin and murinoglobulin (α -1-inhibitor 3) [15,16]. Later, the S form was found to be a mixture of S-1 and S-2 isoforms, having different amino acid sequences in their reactive-site regions [17]. The cDNA clone coding for the F form was identified [18] and the clone for the S-1 form has recently been characterized [19]. In an attempt to isolate the clone for the S-2 form from a rabbit liver cDNA library, a clone coding for a new AP was obtained. The clone encoding the E form showed glutamic acid at the putative P1 site, and the recombinant protein did not inhibit the proteinases. In this paper, the cloning and expression of rabbit APE and the interaction of the recombinant protein with various proteinases are described in comparison with the F and S-1 forms.

EXPERIMENTAL

Materials

Various DNA-modifying enzymes and restriction endonucleases were the products of Takara Shuzo, Toyobo and Promega. Pig pancreatic trypsin, elastase and kallikrein, human plasma thrombin and kallikrein, thermolysin (protease type X), papain and ficin were purchased from Sigma. Bovine chymotrypsin and *Staphylococcus aureus* protease V8 were the products of Cooper Biochemical. Human plasma plasmin and factor Xa, Chromozyme TRY and 5-bromo-4-chloro-3-indolyl phosphate were

Abbreviations used: AP, α -1-antiproteinase; APE, APF and APS-1, the E, F and S-1 forms, respectively, of rabbit AP; IPTG, isopropyl β -D-thiogalactoside; DTT, dithiothreitol.

The nucleotide sequence of α -1-antiproteinase E cDNA has been submitted to the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with accession number D 17725.

obtained from Boehringer Mannheim. Human neutrophil cathepsin G was from Athens Research and Technology. Benzoyl-L-arginine *p*-nitroanilide and succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide were from the Protein Research Foundation (Japan). Benzyloxycarbonyl-leucyl-leucyl-glutamine β -naphthylamide was the product of Bachem Feinchemikalien. Isopropyl β -D-thiogalactoside (IPTG), dithiothreitol (DTT) and Nitro Blue Tetrazolium were from Wako (Japan).

Isolation of the cDNA clone for APE

Since the rabbit AP isoforms were immunologically indistinguishable, and cDNAs encoding the F and S-1 forms have been confirmed, the following two-step screenings were employed [19]. The rabbit liver cDNA library in λ gt11 (Clontech) was screened with rat anti-(rabbit AP) antiserum, and the immunopositive clones were propagated and their DNAs were extracted by standard procedures [20]. Then, probe-1 (5'-GCTGTCTCCTGGCCTCGTC-3'), probe-2 (5'-TGCTATCAGGCAGAGACAT-3') and probe-3 (5'-CACTTTCTGGTAGAGACGAT-3'), complementary to the N-terminal region and the reactive-site regions of the F and S-1 forms respectively, were labelled with 32 P, and those probes were independently dot-hybridized with the isolated cDNAs. The clones which were positive to probe-1, but negative to probe-2 as well as probe-3, were subcloned into phage M13 mp18 and sequenced by the dideoxy method by the Cyclone procedure [18,21].

Construction of the expression vectors and preparation of the recombinant protein

cDNA encoding the E form was propagated in phage M13 and excised with *Hind*III and *Sfi*I, resulting in a 1.4 kbp insert of the E form. Prokaryotic expression vector pkk223-3 (Pharmacia) was digested with *Hind*III and *Eco*RI and ligated with the 1.4 kbp insert, linker-4 (5'-AATTGATATGGACG-3') and linker-5 (5'-CCATATC-3') by use of T4 DNA ligase for 48 h at 12 °C (pkk223-ATE1). *Escherichia coli* JM105 was treated with 50 mM CaCl₂, and the resulting competent cells were transformed with pkk223-ATE1 and plated on agar plates containing ampicillin (50 μ g/ml) and streptomycin (25 μ g/ml). cDNA of the E form was treated with *Sfi*I, and the resulting linear DNA was ligated with linker-8 (5'-CATGGCTGACG-3') and linker-10 (5'-CAGC-3'), followed by digestion with *Hind*III to produce a 1.4 kbp DNA fragment having *Nco*I and *Hind*III sites. This DNA insert was ligated to *Nco*I-*Hind*III-digested pTrc99A (Pharmacia), resulting in expression vector pTrc-ATE1. pTrc-ATE1 transformed the competent cells of *E. coli* JM105 and was plated as above. The third kind of expression vector (pET-ATE1) was prepared by the ligation of the *Nco*I-*Hind*III insert excised from pTrc-ATE1 and pET-21-(d) (Novagen) previously digested with *Nco*I and *Hind*III. pET-ATE1 was used to transform the competent cell BL21 (DE3) and the product was plated on agar plates containing ampicillin (50 μ g/ml). The bacterial colonies for the respective vectors were incubated in L-broth (pkk223-ATE1 and pTrc-ATE1) or 2 $\times$ YT (pET-ATE1) in the presence of 1 mM IPTG for 20 h at 37 °C. Expression of the protein was monitored as described below. A sample of the culture was centrifuged and the precipitate was subjected to SDS/PAGE (15% gels) under reducing conditions. After electrophoresis, the proteins were transferred on to a membrane filter (Immobilon P, Millipore). The membrane was treated successively with 5% skim milk, rat anti-(rabbit antiproteinase) antiserum, and alkaline phosphatase conjugated rat IgG, and stained with a mixture of Nitro Blue Tetrazolium and 5-bromo-

4-chloro-3-indolyl phosphate as described in [20]. Since pET-ATE1 expressed the recombinant protein 50–100 times more than the other plasmids, large-scale preparation was carried out with pET-ATE1. The cells were harvested by centrifugation and then treated with lysozyme and DNase. The crude recombinant protein was purified by chromatography with Superdex 26/60 (Pharmacia), hydroxyapatite (Bio-Rad) and a Mono Q column in f.p.l.c. (Pharmacia). A sample of the eluate from each column was subjected to electrophoresis, and the recombinant E form was detected immunochemically after electroblotting.

Effect of APE on the proteinases

Recombinant APE (0.3 μ g) was allowed to react in 15 μ l of 50 mM Tris/HCl, pH 7.5, for 30 min at 37 °C with various proteinases (0.1–1 μ g), such as trypsin, elastase, chymotrypsin, protease V8, thrombin, factor Xa, plasmin, cathepsin G, plasma and pancreas kallikreins, papain, ficin and thermolysin. To the reaction mixture was added 10 μ l of a sample buffer containing 2% SDS and 0.1 M DTT, and the mixture was heated at 100 °C for 2 min. After being cooled, the sample was subjected to SDS/PAGE (15% gel). The proteins were transferred to a membrane filter and then stained immunochemically as described above.

Assay for proteinase inhibition

Amidolytic activity of trypsin and elastase with chromogenic substrates and proteolytic activity of trypsin with hide powder were measured as described in [12]. The active site of pig trypsin was titrated with *p*-nitrophenyl-*p*-guanidinobenzoate as described by Chase and Shaw [22]. This was used in turn to determine the activity of human α -1-antitrypsin (Sigma), which was then used as a secondary standard for determining the activity of pig pancreatic elastase. The purity of trypsin and elastase was 63.2% and 27.7% respectively. Amidolytic activity of protease V8 was investigated with benzyloxycarbonyl-leucyl-leucyl-glutamine β -naphthylamide in 50 mM Tris/HCl, pH 8.0, at 37 °C [23].

Amino acid sequencing

The recombinant protein (7.5 μ g) was electrophoresed and transferred on to a membrane filter as described above. After being stained with Coomassie Brilliant Blue, the recombinant protein on the filter was subjected to amino acid sequencing with a gas-phase sequencer (470 protein sequencer, Applied Biosystems). The recombinant protein (45 μ g) was incubated with pancreas kallikrein (150 μ g), ficin (3.5 μ g) or protease V8 (15 μ g) in 200 μ l of 50 mM Tris/HCl, pH 7.5, for 30 min at 37 °C. The mixture was loaded on to a reversed-phase column (Chemcosorb 300-7C18; Chemco, Japan) and eluted with a linear gradient of acetonitrile (30–70%) in water containing 0.1% trifluoroacetic acid. A single peak fraction was collected, concentrated and subjected to amino acid sequencing analysis. Recombinant APE (4 μ g) was incubated with various amounts of trypsin (2–10 μ g) for 20 min at 37 °C and subjected to SDS/PAGE under reducing conditions. After electrophoresis, the complex was electroblotted on to Immobilon P and the bands were revealed by staining with Coomassie Brilliant Blue. Six strips corresponding to the respective bands were cut from the membrane and subjected to N-terminal sequencing.

RESULTS

Isolation and characterization of the cDNA for APE

The strategy for isolating the cDNA encoding APE is based on the previous results that the N-terminal regions of the rabbit AP isomers were identical, but the reactive-site regions were highly diverse [12,17–19]. The clone which did not hybridize with probes complementary to the reactive-site region of either the F or S-1 forms of rabbit AP contained 1292 base-pairs, including the open reading frame of 413 amino acids (Figure 1). This clone was termed E isoform, as it has glutamic acid at the putative P1 site, which is considered to be critical in determining the inhibitory

spectrum of AP [24]. The nucleotide sequence and deduced amino acid sequences of the E form are highly similar to those of the F and S-1 forms. The N-terminal half of the amino acid residues is identical for the E and S-1 forms, but 10 out of 15 residues which constitute the reactive-site loop are different between the two forms. The E form has three possible glycosylation sites, the number being the same for the S-1 form but smaller by one than for the F form.

Expression of APE

cDNA for APE was excised from the plasmid DNA and ligated

Figure 1 cDNA and amino acid sequences of rabbit APE

The first 24 amino acids (–24 to –1) represent a putative signal peptide. The reactive-site Glu and three potential carbohydrate-binding sites are shown by ● and ◆ respectively. The residues identified by direct sequencing of the recombinant protein are boxed.

Figure 2 SDS/PAGE of recombinant APE

APE (2.8 μ g) was heated at 100 °C for 2 min in 2% SDS (lane 1) or in 2% SDS with 0.1 M DTT (lane 2) and subjected to SDS/PAGE, followed by staining with Coomassie Brilliant Blue R250. Molecular-mass marker proteins (lane 3) were treated with SDS and DTT: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (1.4 kDa).

to the prokaryotic expression vectors with linker oligonucleotides. Three expression plasmids, pkk223-ATE1, pTrc-ATE1 and pET-ATE1, were constructed to yield a protein whose N-terminal sequence is the same as the sequence of the F and S-1 forms of mature rabbit AP, except for an extension of the dipeptide, methionyl-alanine. ppk223-ATE1 and pTrc-ATE1 have *tac* and *trc* promoters respectively, upstream of the initiation codon, whereas pET-ATE1 has bacteriophage T7 promoter. In the three expression plasmids the E form of rabbit AP was induced with 1 mM IPTG, but pET-ATE1 expressed the highest amount of the target protein, as judged by SDS/PAGE followed by immunochemical analysis. The recombinant protein showed a single band of 40 kDa under reducing conditions in SDS/PAGE, but a band due to dimerization was detected under non-reducing conditions (Figure 2). The purity of monomeric APE was estimated to be 70 %, the value being confirmed by non-denaturing gradient PAGE and h.p.l.c. with a reversed-phase column (results not shown). The N-terminal amino acid sequence of the protein expressed by pET-ATE1 was Ala-Asp-Glu-Ala-Gln-Glu-Thr-Ala-Val-Ser-, confirming the insert cDNA sequence, except for the N-terminal methionine residue that had been efficiently removed.

Interaction of recombinant APE with proteinases

One of the characteristics of AP is its ability to inhibit a wide variety of serine proteinases compared with other serpins that modulate the coagulation cascade, fibrinolysis or complement activation [3,4]. Then the effect of 13 proteinases on recombinant APE was investigated with SDS/PAGE followed by Western blotting, and its inhibitory spectrum of APE on some serine proteinases was compared. The mode of interaction between the E form and proteinases fell into three groups: (i) trypsin and elastase formed SDS-stable complexes with the E form, that gradually degraded; (ii) some of the serine proteinases, such as chymotrypsin, protease V8 and pancreatic kallikrein, and non-serine proteinases, such as papain, ficin and thermolysin, degraded the E form without forming a detectable complex; and (iii) the third group of proteinases, having stringent substrate

Figure 3 Complex-formation of APE with trypsin and elastase

Recombinant APE (0.3 μ g) was incubated for 30 min with various amounts of pig trypsin (lanes 2–4) at 37 °C or pig pancreatic elastase (lanes 5 and 6) at 25 °C. The molar ratio of the proteinase to APE is indicated in parentheses below. The reaction mixture was heated at 100 °C for 2 min with 2% SDS and 0.1 M DTT and subjected to electrophoresis, followed by staining immunochemically as described in the text. Lane 1, APE; lane 2, APE and trypsin (0.5); lane 3, APE and trypsin (1.0); lane 4, APE and trypsin (2.5); lane 5, APE and elastase (0.2); lane 6, APE and elastase (1.0). The relative molecular masses of marker proteins (kDa) are indicated on the right.

specificity, such as thrombin, coagulation factor Xa, plasmin, plasma kallikrein and cathepsin G, did not affect the recombinant APE. As shown in Figures 3 and 4, the E form gave several bands on electrophoresis upon interaction with increasing amounts of trypsin: at 62, 50, 47, 42 and 37 kDa, other than the 40 kDa band of the intact protein. The size of the highest band agreed with the sum of the E form and trypsin (23 kDa), and the smallest one with modified APE whose C-terminal 4 kDa fragment had been released. The intermediate bands may correspond to the complex of modified APE and trypsin. To confirm the above assumption, each complex on the membrane was subjected to sequencing. As summarized in Table 1, two definitive sequences were observed in each sequencing cycle, except for the 40 kDa band of the intact APE. One of the sequences shows the intact N-terminal of APE, and the other sequences are due to trypsin whose N-terminal region was truncated. The 64 kDa band is an equimolar complex of APE and β -trypsin (starting from Ile⁷ of trypsinogen). Both 50 kDa and 47 kDa bands contain α -trypsin (the bond Lys¹³¹–Ser¹³² was cleaved). The 42 kDa and 37 kDa bands are the complexes of trypsin whose Lys¹⁵⁵–Ser¹⁵⁶ and Lys¹⁷⁶–Asp¹⁷⁷ bands, respectively, were cleaved, the latter being known to be pseudo-trypsin [25]. The sizes of the 65 kDa and 50 kDa complexes show good agreement with the calculated values for an equimolar complex with intact APE, but the expected values for the other complexes are larger than the observed value. Therefore, cleavage in the C-terminal region of APE and/or trypsin could have occurred. The N-terminal sequence data of the complexes also showed that the site of interaction or covalent bond is the C-terminal fragment of pseudo-trypsin, but the site for APE is not specified. The E form also formed a complex with pancreatic elastase (lanes 5 and 6 in Figure 3). The complex degraded to a 31 kDa fragment through a smaller number of intermediates than for the complex of the E form with trypsin, indicating that the mode of interaction of elastase toward the E form was different from that of trypsin. The complex-formation of APE with trypsin and elastase was also observed by gradient PAGE under non-denaturing conditions, although the intermediate bands on the gradient gel were not so clear as on the SDS gel (results not shown). The time course of the interaction of trypsin

Table 1 N-terminal sequences of the complexes of trypsin and APE

Two sets of residues for each cycle indicate the complex of APE with β -trypsin (62 kDa) and with truncated trypsin (50, 47, 42 and 37 kDa) as described in the text. The 40 kDa band shows the N-terminal sequence of APE. Numbers in parentheses denote pmol recovered, but they do not necessarily reflect the relative yield of samples of the respective blotted bands, because the bands obtained in several experiments under different conditions have been combined for sequencing as shown in the Experimental section.

Molecular mass of the complex (kDa)	Cycle									
	1	2	3	4	5	6	7	8	9	10
61	A (13)	D (12)	E (10)	A (9)	Q (8)	E (6)	T (4)	A (14)	V (10)	S (1)
	I (12)	V (10)	G (14)	G (9)	Y (9)	T (5)	C (–)*	A (–)†	A (10)	N (5)
50	A (23)	D (20)	E (14)	A (13)	Q (12)	E (9)	T (5)	A (10)	V (7)	S (2)
	S (26)	S (0)	G (4)	S (0)	S (1)	Y (2)	P (6)	S (0)	L (1)	L (2)
47	A (42)	D (38)	E (23)	A (25)	Q (22)	E (19)	T (11)	A (20)	V (16)	S (3)
	S (45)	S (3)	G (11)	S (2)	S (3)	Y (9)	P (15)	S (1)	L (6)	L (5)
42	A (14)	D (12)	E (9)	A (7)	Q (7)	E (8)	T (4)	A (5)	V (3)	S (1)
	S (11)	S (4)	Y (7)	P (9)	G (9)	Q (10)	I (7)	T (5)	G (4)	N (4)
40	A (152)	D (178)	E (149)	A (160)	Q (129)	E (116)	T (92)	A (113)	V (111)	S (30)
37	A (32)	D (11)	E (22)	A (10)	Q (16)	E (14)	T (7)	A (14)	V (13)	S (12)
	D (36)	S (4)	C (–)*	Q (17)	G (11)	D (8)	S (0)	G (9)	G (11)	P (12)

* Cysteine residue was not determined under these running conditions.

† Yield of alanine in this sequence is difficult to assign, due to the same residue being in overlapping sequences.

Figure 4 Time course of complex-formation between APE and trypsin

Recombinant APE (4.8 μ g) and pig trypsin (**a**, 3.2 μ g; **b**, 8 μ g) were incubated in 120 μ l of 50 mM Tris/HCl, pH 7.5, at 37 °C. At the time indicated below, a 15 μ l sample (lanes 1–6) or a 7.5 μ l sample (lane 7) was withdrawn and boiled for 2 min with 5 μ l of sample buffer containing SDS and DTT, and the proteins were revealed after electrophoresis as described in the legend to Figure 3. Lane 1, 0 min; lane 2, 0.25 min; lane 3, 1 min; lane 4, 3 min; lane 5, 10 min; lane 6, 30 min; lane 7, 150 min. The molar ratio of trypsin to APE was 1.0 for (**a**) and 2.5 for (**b**). Relative molecular masses of the bands (kDa) are indicated on the right.

and the E form was examined at two different molar ratios, as shown in Figure 4. At an equimolar ratio, the complex formation was detected in 1 min, and the 42 kDa band was observed after 150 min. On the other hand, in the presence of a molar excess of trypsin, the original band for the E form disappeared in 10 min and most of the complex converged at 37 kDa. The time-course experiment showed that the SDS-stable equimolar complex of the E form with trypsin formed first, followed by successive degradation as mentioned above, and the 37 kDa fragment resisted further degradation.

Effect of recombinant APE on the amidolytic and proteolytic activities of the proteinases

As the E form gave an equimolar complex with trypsin and elastase, and APF, a major isoform in the rabbit plasma, inhibits trypsin and elastase in a stoichiometric manner [12], effects of the E form on these serine proteinases were examined. However, amidolytic activity of trypsin with benzoylarginine *p*-nitroanilide or Chromozyme TRY, or proteolytic activity with hide powder, was not inhibited by the E form at all, even if a several-fold molar excess of the E form was used. Since the complex of APE and β -

trypsin disappeared after incubation for 10 min (Figure 4), the preincubation time was decreased from 15 min to 2 min and then substrate was added. However, the inhibition was not observed. A different preincubation time did not affect the trypsin activity at all. Differently from the S-1 form, which exhibited trypsin-protein amidase activity [12,18], the amidolytic activity of trypsin complexed with the E form was completely abolished by addition of soybean trypsin inhibitor. The amidolytic and proteolytic activities of elastase were not inhibited by the E form. Since the exposed P1 site and the surrounding loop structure of AP afford the complementary domain toward the reactive centre of the target proteinase, and the E form has glutamic acid at the P1 site that could be susceptible to the glutamic acid-specific proteinase, the effect of protease V8 on recombinant APE was examined. The amidolytic activity of protease V8, however, was not affected by the E form.

Confirmation of the amino acid sequence in the putative reactive-site region

Reactive-site amino acids of the F, S-1 and S-2 forms of rabbit AP were confirmed by sequencing the residues in the N-terminal

region of the small peptides digested by trypsin and other proteinases and then overlapping them. After examination of several proteinases, protease V8, pancreatic kallikrein and ficin were employed to split the E form. The fragment was isolated by h.p.l.c. and sequenced. The N-terminal decapeptide of the fragment digested with protease V8 as well as pancreatic kallikrein was Ser-Val-Pro-Asp-Ser-Ile-Thr-Leu-Asp-Arg-, whereas the ficin-derived fragment was Leu-Ile-Pro-Glu-Ser-Val-Pro-Asp-Ser-Ile-. Both sequences agreed with the amino acid residues deduced from the cDNA, and a novel glutamic acid at the putative P1 site was confirmed. It is reasonable that protease V8 cleaved the carboxylic acid side of glutamic acid, but the specificity of kallikrein did not agree with that site. Therefore an excess of enzyme was required to cleave the bond, which is unfavourable for kallikrein.

DISCUSSION

Rabbit APE showed 96 % identity with the F and S-1 forms in 413 residues, including the signal sequence, but recombinant APE inhibited neither elastase nor trypsin. Nevertheless, the E form yielded an equimolar complex with trypsin and elastase, as judged by SDS/PAGE analysis as well as sequencing of the complexes (Figures 3 and 4; Table 1).

The prime role of AP in physiological and pathological conditions is considered to be the inhibition of neutrophil elastase, which is derived from the observation that the decreased level of AP in the plasma results in the destruction of connective tissue of the lung [3,4]. The physiological significance of the E form is not clear, as it has not been investigated whether APE actually occurs in the rabbit plasma. However, non-inhibitory APE having the same overall structure as other inhibitory serpins is interesting from the viewpoint of the reaction mechanism as well as the structure of serpin.

Naturally occurring serpins and recombinant APs having a carboxylic acid at the P1 site have not been reported, except for cowpox-virus serpin, which inhibits cysteine proteinase [1,26]. Figure 5 shows the amino acid sequence in the reactive-site region of mammalian APs deduced from their cDNAs. Every species has methionine at the P1 site, and some mammals have multiple isoforms whose P1-site residues are different from methionine. Therefore, the methionyl group at the reactive site confers the indispensable role in modulating the proteinase activity *in vivo* [3,4,19], and other APs, not with active-site

methionine, may have an alternative function which has not been uncovered. Numerous point mutations of human AP have been found, but the change in the P1 site is rare. A striking example is the Pittsburg mutant, in which the P1-site methionine was replaced by arginine, and the mutant changed from an elastase inhibitor to a thrombin inhibitor [34]. On the other hand Jallat et al. [35] prepared several recombinant APs whose P1 site was replaced by hydrophobic amino acid residues, and showed that the P1-site amino acid replacement could not always alter the inhibitory spectrum. Guinea-pig plasma contains two isoforms of AP (F and S), and their P1-site residues are histidine and methionine, respectively [29]. The two isoforms, however, inhibited trypsin, chymotrypsin and elastase in a stoichiometric manner [36]. Rubin et al. [37] prepared a recombinant human α -1-antichymotrypsin variant whose P1 residue was changed from leucine to methionine, resulting in the loss of inhibitory activity against elastase. This variant, however, formed an SDS-stable complex with elastase in a manner similar to recombinant APE. Differently from APE, the recombinant variant was a substrate for trypsin and did not form an SDS-stable complex with trypsin. Three rabbit APs have a sequence from P9 to P29 in common, and the hinge region (P10–P14) is the same as in other inhibitory serpins, suggesting that they could interact with proteinases. The different amino acid residues from P8 to P'8 among the three isoforms are chemically alike, except for the P1 site, which could be responsible for the conversion of APE from the elastase inhibitor into a non-inhibitory serpin (Figure 5).

We have shown that the catalytic centre of trypsin complexed with the S-1 form was intact, and a low-molecular-mass substrate could reach it, but the access of soybean trypsin inhibitor was hindered, resulting in trypsin-protein amidase activity [12,14]. We also showed that recombinant APS-1 inhibited elastase and exhibited trypsin-protein amidase activity [19]. Therefore both APE and APS-1 interacted with trypsin in a similar manner, except that soybean trypsin inhibitor could approach the catalytic centre of trypsin complexed with APE, but not with APS-1. Although it is not clear whether the same amino acid residue of the E and S-1 forms participates, the covalent linkage toward trypsin would occur at a site different from the reactive-site seryl residue and other residues that confer the enzyme activity. The P1–P'1 bond of the S-1 form was attacked by trypsin [17], but the initial stage of the interaction of the E form with trypsin is not known. It is necessary to determine the site of covalent linkage between the E or S-1 form with trypsin for the thorough understanding of the inhibition mechanism of rabbit APs. It is also interesting to know whether the E form actually occurs in the rabbit plasma and has a physiological function. Research along these lines is being carried out in our laboratory.

Recently, the clone coding for the S-2 form was isolated by screening an acute-phase cDNA library of rabbit liver using human α -1-antitrypsin cDNA as probe [38]. The deduced amino acid sequence of the reactive-site region, including tyrosine at the P1 site, agreed with the sequence that had been reported by us [17], and the recombinant APS-2 inhibited elastase and chymotrypsin. The E isoform, however, was not included in the clones that have been selected for sequencing.

	P ₁₅	P ₁₀	P ₅	P ₁	P' ₁	P' ₅	
Human	G	T	E	A	A	G	A
Bovine	G	T	E	A	A	G	A
Sheep	G	T	E	A	A	G	A
Rabbit F	G	T	E	A	A	G	A
Guinea pig S	G	T	E	A	A	G	A
Rat	G	T	E	A	A	G	A
Mouse	G	T	E	A	A	G	A
Mongolian gerbil	G	T	E	A	A	G	A
Opossum	G	T	E	A	A	G	A
Guinea pig F	G	T	E	A	A	G	A
Rabbit S-1	G	T	E	A	A	G	A
Rabbit E	G	T	E	A	A	G	A

Figure 5 Comparison of the reactive-site amino acid sequences of mammalian APs

The amino acid sequences of AP from human [5], bovine [27], sheep [28], rabbit (F [18], S-1 [19] and E (this paper)), guinea pig (F and S) [29], rat [30], mouse [31], Mongolian gerbil [32] and opossum [33] are deduced from the respective cDNAs. The residues which are the same for more than 10 out of 12 are boxed.

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