The Contribution of the Conserved Hinge Region Residues of α_1-Antitrypsin to Its Reaction with Elastase†

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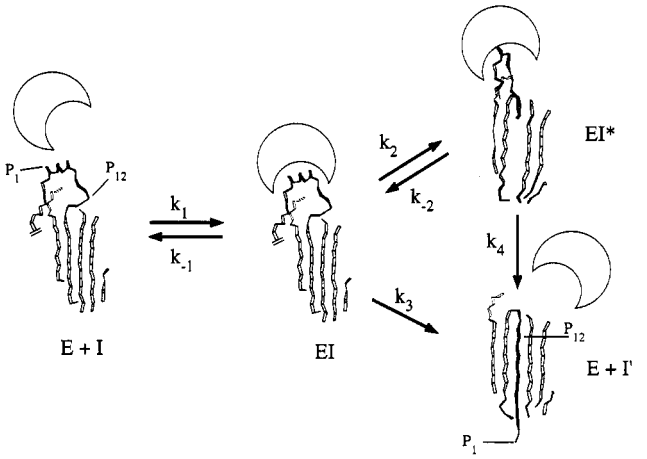
ABSTRACT: The hinge region of serpins is a conserved sequence of 8 amino acids located 7 residues away from the scissile bond at P_8 to P_{15}, on the edge of the protease-binding domain. In the inhibitory serpins the P_8 to P_{12} residues of this motif are usually small side-chain amino acids, most commonly alanine. Each of these residues in α_1-antitrypsin was mutated to a glutamate, and the effect of the mutation on the inhibitory characteristics was assessed. A strong positional dependence of the effect of a hinge-region glutamic acid substitution was found. While substitutions at positions P_{10} and P_{12} affected the inhibitory characteristics of α_1-antitrypsin, substitutions at positions P_7, P_8, P_9, and P_{11} had no effect on inhibition. Thus, the conservation of residues with small side chains at the latter positions does not appear to be related to an essential function in the inhibitory mechanism. Following the glutamate substitution at P_{10}, α_1-antitrypsin remained a rapid inhibitor of elastase, but the elastase−serpin complex slowly broke down to yield active elastase and cleaved α_1-antitrypsin. The glutamate substitution at P_{12} caused the resultant molecule (P_{12} Ala → Glu) to become a partial substrate of elastase such that four moles of inhibitor were required to inhibit one mole of enzyme, and led to a 12-fold decrease in the association rate constant. The data could be interpreted in terms of the suicide substrate inhibition model for serpin−protease interactions and allowed a further refinement of the role of the hinge region in this process.

Serpins are a large family of proteins sharing a common fold typified by the structure of α_1-antitrypsin (α_1-AT).[1] The conserved core of the serpin consists of 8 α-helices arranged around 3 β-sheets (Löbermann et al., 1984). The reactive-site loop, which connects strand 5 of the A β-sheet to strand 1 of the C β-sheet (Carrell et al., 1994; Stein et al., 1990; Wei et al., 1994), forms a very stable complex with the target protease. Since the first serpin structure was solved (Löbermann et al., 1984), it has been known that a large structural transformation [the S to R transition (Carrell and Owen, 1985)] takes place when the reactive-site loop is cleaved. This involves the insertion of the reactive-site loop as an extra, central, strand into the A β-sheet (I′, Scheme 1). This insertion is accompanied by a large increase in structural stability caused not only by the creation of the extra strand into the A β-sheet, but also by many smaller-scale improvements in the order of the whole molecule (Gettins, 1989; Perkins et al., 1992).

While the majority of the serpin family are inhibitors of serine proteases, a number of members fail to function as inhibitors; two of the noninhibitors, ovalbumin and angiotensinogen, also fail to undergo the S to R transition (Stein et al., 1989). Comparison of the amino acid sequence of the inhibitor members of the family with that of the noninhibitors shows that one particular region, which borders the enzyme-binding region, is highly conserved in the inhibitor members of the family, yet not in the noninhibitors (Table 1), suggesting an involvement with the S to R transition and the inhibition process. It is assumed that a structural change resembling the S to R transition accompanies the formation of the serpin−enzyme complex,

Scheme 1: Scheme Combining a Minimal Description of the Suicide Substrate Mechanism with Current Knowledge of Serpin Structural Variation[a]



[a] For the purpose of clarity, only the reactive-site loop, strand 1 C, and the A β-sheet are shown. The structure of the intact serpin (I) is modeled on ovalbumin (Stein et al., 1990) and closely resembles that of the intact structure of the inhibitory serpin α_1-antichymotrypsin (Wei et al., 1994). The structure of the cleaved serpin (I′) is that of α_1-AT (Löbermann et al., 1984). The structures of both enzyme−inhibitor complexes (EI and EI*) are unknown, but data from previous studies suggest that they are feasible (see main text).

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[1] Abbreviations: α_1-AT, α_1-antitrypsin (also known as α_1-proteinase inhibitor); ΔN15, α_1-AT with the 15 amino-terminal amino acids removed; BSA, bovine serum albumin; SI, stoichiometry of inhibition. Residues in the reactive-site loop at α_1-AT are defined according to Schechter and Berger (1967): P_{n+1}, P_n, ... P_1, P'_1 ... P'_n, P'_{n+1}, where the $P_1-P'_1$ bond is the scissile bond ($Met^{358}-Ser^{359}$ in α_1-antitrypsin). Hinge region substitutions are described by their position and the substituted amino acid; e.g., $P_{12}E$ is α_1-AT with a P_{12} Ala to Glu substitution (Ala^{347} → Glu).

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Table 1: An Alignment Showing Conservation of the Hinge Region Consensus Sequence in Inhibitory Serpins but Not in Noninhibitory Serpins[a]

residue position:	P_{15}	P_{14}	P_{13}	P_{12}	P_{11}	P_{10}	P_9	P_8	P_7
consensus sequence:	G	T	E	A	A	A	A	T	A
inhibitors	100[b]	77	61	95	72	55	55	77	30
noninhibitors	80	20	80	20	40	20	0	20	0

[a] The hinge regions of 21 inhibitors serpins and 5 noninhibitor serpins were aligned by hand; [b] Percent absolute conservation of serpin hinge region consensus sequence. Source: The sequences were taken from Marshall (1993).

as it has structural features in common with the cleaved structure that are not shared with the unassociated, intact serpin (de Agostini et al., 1985; Björk et al., 1993; Munch et al., 1991). Specifically, it is thought that partial insertion of the hinge region into the A β-sheet is required for the inhibitory conformation of the reactive-site loop (see EI*, Scheme 1). Indeed, point mutations of the hinge region commonly lead to an increased proportion of proteolysis of the reactive site loop by the target protease (Holmes et al., 1987; Hood et al., 1994; Hopkins et al., 1993; Perry et al., 1989; Schulze et al., 1991; Skriver et al., 1991).

A minimal reaction pathway for the serpin–enzyme reaction is depicted in Scheme 1. Similar schemes have been previously proposed (Cooperman et al., 1993; Olson, 1985; Patston et al., 1991). After the formation of the initial complex (EI), the reaction may proceed along two divergent paths, one leading to the formation of a stable enzyme–inhibitor complex (EI*) and the other to reactive-center cleaved serpin (I′) and active enzyme (E). The nature of the stable complex is still in dispute, and may resemble the tetrahedral intermediate in the catalysis of peptide substrates by serine proteases (Matheson et al., 1991). The stable complex may then break down slowly to release inactive inhibitor and enzyme, either through an extension of the inhibitory pathway (Cooperman et al., 1993; Patston et al., 1991) or through its reversal, as shown in Scheme 1. If it is assumed that an S to R-like transition and insertion of the hinge region into the A β-sheet are essential for the formation of the stable serpin–enzyme complex (EI*), the effect of blocking this transition would be to favor proteolytic cleavage of the reactive-site loop (i.e., formation of I′). Examination of the crystal structure of α_1-AT (Löbermann et al., 1984) and other serpins (Carrell et al., 1994; Delarue et al., 1990; Mottonen et al., 1992) indicates that substitution of larger or charged amino acids in the hinge region would hinder, or raise the energy barrier for, strand insertion. Previous data (Hood et al., 1994; Lawrence et al., 1994; Schulze et al., 1991; Skriver et al., 1991) have clearly demonstrated that mutagenesis of the P_{14} and P_{12} residues, especially to charged amino acids, interferes with normal inhibition behavior. The conserved sequence of the hinge region, however, includes residues from P_8 to P_{15}. In order to address the role of this region in both the S to R transition and in inhibition, each residue of the hinge region of α_1-AT from P_7 to P_{12} was separately mutated to a glutamic acid, and the effects of the substitutions determined.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA), and oligonucleotides synthesized by members of the Department of Biochemistry, University of Cambridge. Polyclonal rabbit antibodies against human α_1-AT, TPCK-treated bovine pancreatic trypsin, papain, *p*-nitrophenyl guanidinobenzoate and *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide were obtained from Sigma (St. Louis, MO, USA). Alkaline phosphatase–conjugated goat anti-rabbit antibodies and Bradford assay reagents were purchased from Biorad (Hercules, CA, USA), and the BCIP/NPT alkaline phosphatase substrate kit was purchased from Vector Laboratories (Peterborough, UK). D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) was obtained from Kabi-Pharmacia (Uppsalla, Sweden). *Escherichia coli* BL21-(DE3) was obtained from F. William Studier, Brookhaven National Laboratories (Upton, NY, USA). Rifampicin was obtained as Rifadin (Merryl Dow, NJ, USA). Human leukocyte elastase was a gift of Drs. David Bruce and Mark Wardell (Dept. Haematology, University of Cambridge, England). Other reagents were of the highest grade commercially available.

Production and Purification of α_1-AT Variants in E. coli. The first 15 codons deleted in the construction of pΔN15 were removed by digestion of pTermat (Hopkins, et al., 1993) with Bcl I and Bam HI after growth in *E. coli* JM110, and religation of the compatible overhangs. Site-directed mutagenesis was performed using PCR-based techniques. Regions amplified by the PCR were sequenced to confirm the existence of the desired mutation and to exclude the possibility of Taq-induced error.

The proteins were expressed in *E. coli* essentially as previously described (Hopkins et al., 1993), except that rifampicin was omitted when using pΔN15. Mutants P_{10}E, P_{11}E, and P_{12}E were produced in pΔN15 whereas pTermat was used for P_7E, P_8E, and P_9E. With the exception of P_{11}E, the proteins were purified as previously described (Hopkins et al., 1993). It was not possible to purify P_{11}E with this protocol, or other published protocols. Thus, experiments with P_{11}E were carried out on protein partially purified as follows: 10 g of cell paste was lysed in a French Press in 300 mM NaCl, 50 mM Tris-HCl at pH 8.0, and 5 mM EDTA. The lysate was treated with poly(ethylene glycol) of M_r 8 000, at 8% (w/v) for 1 h at 4 °C and centrifuged at 20 000*g* for 40 min. Further poly(ethylene glycol) of M_r 8 000 was added to the supernatant to achieve a final concentration of 40% w/v. After stirring at 4 °C for 1 h, the solution was centrifuged at 20 000*g* for 40 min, the pellet was resuspended in 50 mM Tris-HCl at pH 8.0, containing 2 mM EDTA and loaded onto a 1-mL Mono-Q column. Bound protein was eluted with a 50–250 mM NaCl gradient in 50 mM Tris-HCl at pH 8.0. The α_1-AT-containing fractions, identified by rocket immunoelectrophoresis, were pooled and stored at −80 °C after snap-freezing in liquid nitrogen.

S to R Transition. Papain was used to cleave α_1-AT and mutants in the reactive-site loop (Mast et al., 1992). The optimal conditions for cleavage of each protein were determined by incubation with increasing amounts of papain for 30 min at 37 °C. The reactions were terminated by the addition of 5 mM iodoacetamide, and a change in migration of the protein on SDS–PAGE was used to confirm the cleavage. The heat stabilities of native and reactive-site loop-cleaved α_1-AT were assayed by heating the samples between 25 and 100 °C for 2 h in 75 mM Tris, 75 mM glycine, 75 mM NaH_2PO_4 buffer at pH 8.0, followed by centrifugation

for 20 min at 10 000*g*. Residual soluble protein was assayed by rocket immunoelectrophoresis (Stein et al., 1989).

Determination of the Concentration of Proteins. The protein concentration of each preparation was determined by Bradford assay against a standard of purified recombinant α_1-AT whose concentration had been determined by amino acid analysis. The concentration of α_1-AT in the $P_{11}E$ preparation was determined by rocket immunoelectrophoresis using serial dilutions of α_1-AT ΔN15 as a control. The concentration of elastase was determined by titration against α_1-AT whose concentration was determined both by protein concentration and by titration against trypsin, whose concentration was determined by active-site titration with *p*-nitrophenyl *p'*-guanidinobenzoate according to the method of Chase and Shaw (1970).

The inhibitory titer of each α_1-AT sample was determined against elastase in 30 mM phosphate buffer, at pH 7.4, containing 160 mM NaCl, 0.1% (w/v) poly(ethylene glycol) (M_r 6 000) and 0.1% (v/v) Triton X-100. Reactions were performed in BSA-coated microtiter plates. Elastase (5.32 nM) was incubated with increasing amounts of inhibitor in a volume of 100 μL at room temperature for 20 min, and the residual activity of the enzyme was determined by adding 100 μL of buffer containing the chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. Linear regression analysis of the data was used to determine the concentration of inhibitor present in the sample.

The stoichiometry of inhibition (SI) was estimated by dividing the serpin titer expected from the Bradford assay or rocket immunoelectrophoresis estimation by that obtained by titration against elastase.

Kinetic Assays. All kinetic measurements were performed at 37 °C in 30 mM phosphate buffer, at pH 7.4, containing 160 mM NaCl, 0.1% (w/v) poly(ethylene glycol) (M_r 6 000), and 0.1% Triton X-100, and reactions were performed in BSA-coated cuvettes. The determination of kinetic parameters by progress curve kinetics was performed as previously described (Hopkins et al., 1993; Stone & Hofsteenge, 1986) with elastase concentrations of 0.1−0.3 nM. Inhibitor concentrations were 0.4 to 300.0 nM. Each progress-curve experiment consisted of 7 assays, one in which the inhibitor concentration was zero, and six with different inhibitor concentrations. Analysis of the data by nonlinear regression yielded estimates of the association constant (k_{ass}). The K_i for the inhibitors could not be determined under the conditions used; however, considering the conditions of the experiments, an upper limit of 1 pM could be placed on all reactions. Progress-curve experiments were performed at least twice, and the values reported represent the weighted mean of the determinations. Values of K_m required for the calculation of k_{ass} were determined by standard initial-velocity studies; an estimate of 85.5 ± 2.5 μM was obtained for *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide with human leukocyte elastase.

Analysis of Reaction Products by SDS−PAGE. α_1-AT variants, 0.4 μM as determined by inhibitory titer, were incubated with an equal concentration of elastase in a volume of 20 μL at 25 °C for 2 min. SDS-gel loading buffer (5 μL) containing 2.5% SDS was added and the sample heated at 100 °C for 3 min before analysis by SDS−PAGE on a 10% acrylamide gel. In the case of $P_{11}E$, the gel was analyzed by Western blotting. The time-course of the reaction of elastase with $P_{10}E$ was performed by incubating 0.5 μM elastase with 1.0 μM $P_{10}E$ in 160 μL of 30 mM phosphate buffer, at pH 7.4, containing 160 mM NaCl, 0.1% (w/v) poly(ethylene glycol) (M_r 6 000), and 0.1% Triton X-100; nine 8-μL aliquots were removed at the indicated times, combined with SDS−PAGE loading buffer and frozen at −80 °C. The remainder of the material was used to determine the cleavage site as described below.

Determination of Cleavage Sites. The cleavage site of the serpins by the proteases was determined as previously described (Pemberton et al., 1988). Briefly, an equal volume of 8 M guanidinium hydrochloride was added to the serpin−protease sample before fractionation by reverse-phase HPLC on a PLRP column (Polymer Laboratories, Shropshire, UK) using an acetonitrile gradient in 0.1% trifluoroacetic acid. The C-terminal fragment eluted wth 50% acetonitrile, and the larger N-terminal fragment eluted at 64% acetonitrile; the samples were collected and subjected to N-terminal sequencing (Dr. Len Packman, Dept. of Biochemistry, University of Cambridge).

RESULTS

Expression and Purification of Mutant Proteins. The level of expression of the native α_1-AT sequence in *E. coli* is poor, because of a less than optimal structure of the ribosome binding site (Johansen et al., 1987; Sutiphong et al., 1987). The present study found that the mutation of the P_{10} Gly to Glu virtually abolished expression of α_1-AT using the vector pTermat. The cDNA for $P_{10}E$ was therefore cloned into pΔN15, which is missing the first 15 codons of α_1-AT; this has been previously shown to improve expression levels (Johansen et al., 1987; Sutiphong et al., 1987). This cloning resulted in expression levels of $P_{10}E$-ΔN15 that were indistinguishable from the wild-type protein with its 15 N-terminal codons removed (ΔN15). As a consequence of this observation, the variants $P_{10}E$, $P_{11}E$, and $P_{12}E$ were all expressed in pΔN15; little variation was seen in the levels of expression of all these variants. The production of variants in pΔN15 required slight alterations to the expression method as rifampicin caused all the protein to be deposited as inclusion bodies. Proteins were purified as previously described (Hopkins et al., 1993). At no point was any difference observed between the functional characteristics of ΔN15 and the wild-type protein having an intact N-terminus.

The $P_{11}E$ mutation had effects on the ease of purification. This variant could not be purified in the usual manner, in spite of repeated attempts. The purification difficulties were not due to low expression levels; these were not noticeably different from the wild-type ΔN15 protein, as determined by rocket immunoelectrophoresis of bacterial lysates. Consequently, all assays with $P_{11}E$ were conducted with partially purified material (30−60% pure as determined by Coomassie-stained SDS−PAGE).

S to R Transition. The mutants P_7E and P_8E could not be cleaved by papain solely or mainly within the reactive-site loop. Mast et al. (1992) have shown that the primary cleavage site for papain in the α_1-AT reactive-site loop is between the P_7 phenylalanine and the P_6 leucine with a secondary site between residues P_1 and P'_1. Sequences close to the primary cleavage site were mutated in the P_7E and P_8E variants cleavage at this site may be affected but it was anticipated that cleavage would still occur at the $P_1-P'_1$

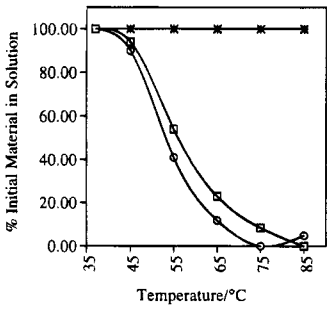

FIGURE 1: S to R transition assay of α_1-AT hinge region variants. α_1-AT and $P_{10}E$, either uncleaved or cleaved with papain were heated at the range of temperatures indicated for 2 h. Residual soluble protein was assayed by rocket immunoelectrophoresis after centrifugation. Uncleaved wild-type recombinant α_1-AT is represented by □, and uncleaved $P_{10}E$ is represented by ○. Cleaved wild-type recombinant α_1-AT is represented by ×, and cleaved $P_{10}E$ is represented by +.

secondary site. However, it was not possible to determine conditions that resulted in the majority of cleavage occurring at the secondary site at $P_1-P'_1$. This site was no more susceptible to cleavage than other sites in α_1-AT. Presumably in wild-type α_1-AT, the P_6 to P_7 bond is cleaved first, followed by the S to R transition which results in the protection of other papain cleavage sites except the exposed $P_1-P'_1$ bond. All other variants were cleaved within the reactive-site loop by papain.

The heat stabilities of intact and papain-cleaved proteins were determined. Figure 1 shows the residual soluble protein for cleaved and uncleaved proteins after incubation between 25 °C and 100 °C for ΔN15 and $P_{10}E$; similar data were obtained for the other variants. The data indicated that cleavage within the reactive-site loop led to the marked increase in heat stability typical of inhibitory serpins.

The Inhibitory Properties of the Variants. The inhibition of elastase by α_1-AT can be analyzed in terms of the suicide substrate mechanism presented in Scheme 1. After the formation of an initial complex, the reaction partitions between two pathways, one leading to the formation of a stable enzyme−inhibitor complex and the other leading to the formation of cleaved serpin (Cooperman et al., 1993; Fish & Björk, 1979; Hood et al., 1994; Hopkins et al., 1993; Patston et al., 1991). The ratio of the rate constants for these two steps (k_3/k_2) is known as the *partition ratio*, defined as r (Waley, 1985); the stoichiometry of inhibition (SI) is equal to $1 + r$. This ratio can be calculated by titration experiments in which a constant amount of enzyme (E_0) is incubated with increasing amounts of inhibitor; a plot of enzyme activity against inhibitor concentration will yield a straight line which intercepts the abscissa at $E_0.SI$. The cleavage pathway (k_3) leads to the depletion of the inhibitor; however, Waley has shown that under the conditions of the progress-curve assays, the cleavage pathway can be ignored, provided that $I_0 \gg SI.E_0$ (Waley, 1985). In these circumstances the reaction may be conveniently treated as having a linear pathway, and the association rate constant and inhibition constant may be calculated using progress curves as previously described (Hopkins et al., 1993; Stone & Hofsteenge, 1986).

Stoichiometry of Inhibition. Table 2 gives the value of SI for each variant tested, most of the variants had an SI value of less than 1.5. Only $P_{12}E$ had a significantly higher value of SI (4.2) against elastase.

Table 2: The Effect of Hinge Reaction Mutations on the Stoichiometry of Inhibition (SI) and on the Association Rate Constant[a]

	$k_{ass}/M^{-1}\ s^{-1}$	SI	$k_{ass} \times SI$
	Trypsin		
wild-type[b]	$(2.21 \pm 0.06) \times 10^5$	1.2	$(2.65 \pm .07) \times 10^5$
P_{10}Pro[b]	$(0.97 \pm 0.03) \times 10^5$	5.6	$(5.43 \pm .17) \times 10^5$
	Human Leukocyte Elastase		
wild-type[c]	$(1.20 \pm 0.05) \times 10^7$	—	—
ΔN15[c]	$(1.24 \pm 0.03) \times 10^7$	—	—
P_7E	$(1.40 \pm 0.77) \times 10^7$	1.3	$(1.82 \pm 1) \times 10^7$
P_8E	$(0.82 \pm 0.08) \times 10^7$	1.2	$(0.98 \pm .1) \times 10^7$
P_9E	$(0.75 \pm 0.05) \times 10^7$	1.4	$(1.05 \pm .07) \times 10^7$
$P_{10}E$	$(1.41 \pm 0.02) \times 10^7$	1.0	$(1.41 \pm .02) \times 10^7$
$P_{11}E$	$(0.66 \pm 0.12) \times 10^7$	1.5	$(1.0 \pm .18) \times 10^7$
$P_{12}E$	$(0.10 \pm 0.01) \times 10^7$	4.2	$(0.42 \pm .04) \times 10^7$
P_{10}Pro[b]	$(6.20 \pm 0.01) \times 10^4$	160	$(0.99 \pm .01) \times 10^7$
	Porcine Pancreatic Elastase		
wild-type[d]	2.8×10^5	1	2.8 ± 10^5
$P_{14}R$[d]	1.6 ± 10^3	70	1.12×10^5

[a] Association rate constants and SI values were determined as described in Materials and Methods. [b] Hopkins (1993). [c] The SI value of wild-type and ΔN15 with elastase could not be determined since the concentration of elastase was determined by titration against these proteins. [d] Hood (1994)

Progress-Curve Kinetics. The inhibition of elastase was examined by progress-curve kinetics in the presence of chromogenic substrate, and the data fitted to the equation for slow tight-binding inhibition (Hopkins et al., 1993; Stone & Hofsteenge, 1986). The results are given in Table 2. Most of the mutations had little effect on the association rate constants with elastase. $P_{12}E$ was the exception; the k_{ass} value for $P_{12}E$ with elastase was 12-fold lower than that for the other α_1-AT variants. In spite of its high SI value, the complexes formed with $P_{12}E$ were as stable as those formed with wild-type α_1-AT (compare the data presented in Figure 2B with those obtained with wild-type shown in Figure 2A). The results with the $P_{10}E$ variant were, however, different. Although the $P_{10}E$ variant had an SI value that was not notably different from the wild-type, longer incubations of $P_{10}E$ with elastase showed a return of enzyme activity (Figure 2C). Only data acquired before 75 min were used for the calculation of kinetic constants for $P_{10}E$.

Analysis of Reaction Products. The products of the reactions between the variants and elastase were analyzed by SDS−PAGE. Serpin and elastase were incubated at 1:1 molar ratios. The serpin concentration was based on the stoichiometry of inhibition; i.e., $(1 + r)$ moles of serpin were present for each mole of protease. Figure 4 shows that all of the variants were able to form SDS−stable complexes with elastase. All variants also produced a small amount of cleaved inhibitor, which accounts for the slightly elevated SI values seen.

The variant $P_{12}E$ had the highest SI value calculated, and Figure 4B (lane 4) shows that this can be accounted for by the coincident generation of cleaved inhibitor, which is consistent with the suicide substrate model of serpin inhibition. Sequencing of the products of the reaction between $P_{12}E$ and elastase confirmed that cleavage had occurred at the $P_1-P'_1$ bond.

Longer incubations of $P_{10}E$ with elastase showed a return of enzyme activity (Figure 2C). The products of the reaction were investigated by incubating elastase and $P_{10}E$ at a 1:2 molar ratio (0.5 and 1.0 μM, respectively) at 37 °C. Samples

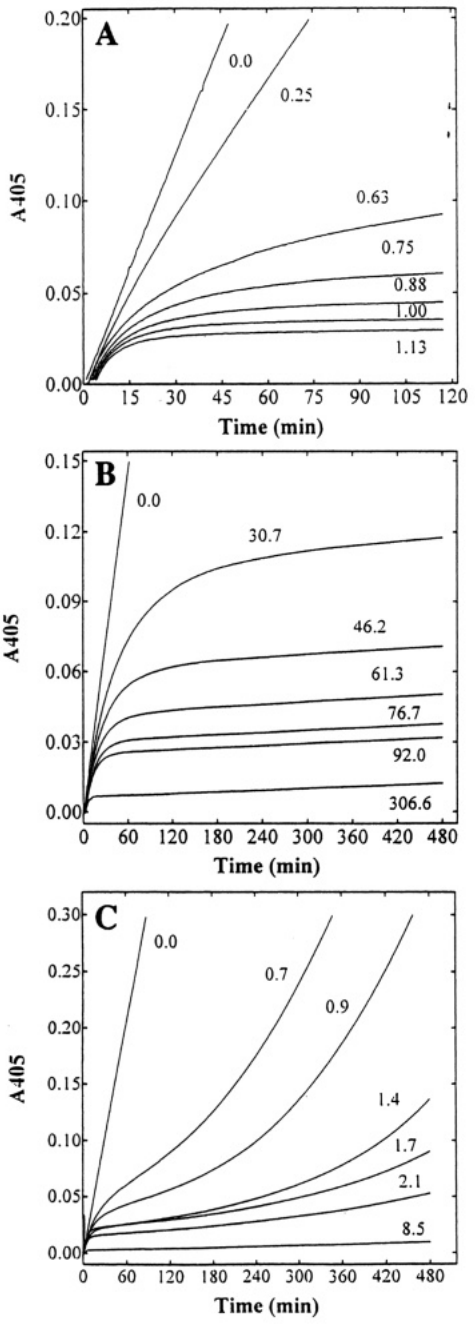

FIGURE 2: Progress curves for the inhibition of elastase by wild-type recombinant α_1-AT (A), $P_{12}E$ (B), and $P_{10}E$ (C) at 37 °C. The amount of free enzyme was monitored through the conversion of *p*-nitroanilide to *p*-nitroaniline, monitored at 400−410 nm. The amounts of enzyme, inhibitor, and chromogenic substrate are as follows: (A) 0.11 nM elastase was incubated with increasing concentrations of α_1-AT at (in clockwise order) 0, 0.25, 0.63, 0.75, 0.88, 1.0, and 1.13 nM, respectively, in the presence of 490 μM N-MeOH-Suc-Ala-Ala-Pro-Val-*p*NA. (B) Progress curve for the inhibition of elastase by $P_{12}E$. 0.27 nM elastase was incubated with increasing concentrations of $P_{12}E$ at (in clockwise order) 0, 30.7, 46.2, 61.3, 76.7, 92.0, and 306.6 nM, respectively, in the presence of 530 μM N-MeOH-Suc-Ala-Ala-Pro-Val-*p*NA. (C) Progress curve of $P_{10}E$ incubated with elastase, showing regain of enzyme activity: 0.27 nM elastase and increasing concentrations of $P_{10}E$ at (in clockwise order) 0, 0.7, 0.9, 1.4, 1.7, 2.1, and 8.5 nM, respectively, were incubated in the presence of 490 μM N-MeOH-Suc-Ala-Ala-Pro-Val-*p*NA.

were removed at timed intervals, SDS-gel loading buffer was added, and the samples were stored at −80 °C. When all samples had been collected, they were subjected to SDS−PAGE. Figure 3 shows the appearance of cleaved material. HPLC fractionation of material identical to that in lane 10 yielded two peaks, one corresponding to the C-terminus of α_1-AT that would be generated by cleavage within the reactive-site loop; sequencing of this peak yielded the sequence Ser, Ile, Pro, Pro, Glu, confirming that α_1-AT $P_{10}E$ had been cleaved at the $P_1-P'_1$ bond. The second peak yielded a sequence corresponding to the N-terminus of the intact protein. Thus, the $P_{10}E$ variant initially forms complexes with elastase that are indistinguishable from normal complexes (Figures 3 and 4). These complexes, however, dissociate more rapidly than wild-type α_1-AT into active enzyme (Figure 2C) and reactive-center-cleaved serpin (Figure 3).

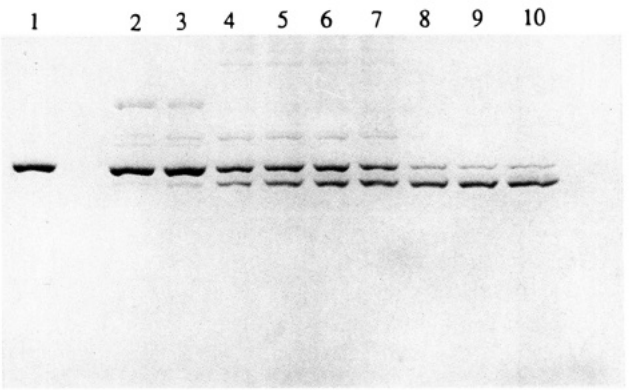

FIGURE 3: SDS−PAGE (10% acrylamide) showing the generation of cleaved $P_{10}E$ on incubation with elastase. 0.5 μM elastase was incubated with 1.0 μM $P_{10}E$ at 37 °C, samples were removed at various times and denatured with SDS-gel loading buffer at 100 °C. *Lane 1*: prior to the addition of elastase; *lanes 2−10*: 5, 100, 1 550, 2 040, 2 835, 4 525, 5 645, 7 200, and 11 675 min. after the addition of elastase, respectively. The material used to identify the cleavage site is the same as that in lane 10.

DISCUSSION

In the current study, the effect of substituting the small side-chain amino acids of the hinge region of α_1-AT from P_7 to P_{12} with glutamic acid has been examined. A strong positional dependence of the effect of the mutation was observed. The P_7E, P_8E, P_9E, and $P_{11}E$ mutants were close to wild-type protein with respect to the stability of the complexes formed and the partition ratio. In contrast, the $P_{12}E$ mutation caused an increase in the partition ratio. Although the $P_{10}E$ mutation did not affect the k_{ass} value or partition ratio, the complex of this mutant with elastase slowly broke down to release cleaved serpin.

None of the mutants tested prevented the S to R transition. The results with $P_{12}E$ contrast with those obtained by Skriver et al. (1991); however, their results were based on immunological data using an antibody that specifically recognizes the cleaved form of C1-inhibitor. It is possible that the assay used here does not identify subtle changes in structure that the immunological methods of Skriver et al. (1991) recognized. Nevertheless, in conjunction with other studies (Hood et al., 1994; Hopkins et al., 1993; Lawrence et al., 1994; Wright & Blajchman, 1994), our results demonstrate that cleaved hinge region variants still undergo a dramatic increase in stability upon cleavage, and that small amino acids in the hinge region are not an absolute requirement for the S to R transition. Examination of cleaved serpin structures (Baumann et al., 1991; Delarue et al., 1990;

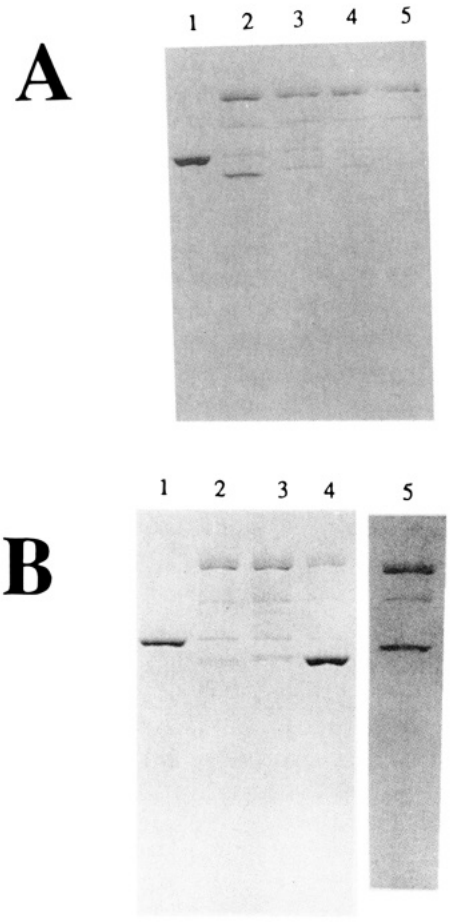

FIGURE 4: Analysis of the reaction products arising from the incubation of hinge region variants with elastase by 10% acrylamide SDS−PAGE. (A) Proteins produced with the vector pTermat. *Lane 1*: unreacted wild-type α_1-AT; *lanes 2−5*: wild-type, P_7E, P_8E, and P_9E, respectively, reacted with an equimolar amount of elastase for 2 min at room temperature. (B) Proteins produced with the vector pΔN15. *Lane 1*: unreacted ΔN15; *lanes 2−4*: ΔN15, $P_{10}E$, and $P_{12}E$ reacted with equimolar amounts of elastase for 2 min at room temperature; *lane 6*: Western blot of $P_{11}E$ reacted with an equimolar amount of elastase for 2 min at room temperature.

Löbermann et al., 1984) indicates that a glutamate could not be stably inserted into the A β-sheet. Thus with these mutants, insertion of the entire reactive site loop into the A β-sheet is unlikely. It has been demonstrated that the increase in stability upon insertion of the reactive-site loop and hinge region is caused not only by the creation of an additional β-sheet strand but also by a general optimization of secondary structure (Gettins, 1989; Haris et al., 1990; Perkins et al., 1992). The wider secondary structure changes are initiated by the insertion of the reactive-site loop into the A-sheet and its subsequent expansion. Although in the absence of more detailed structural data, for example of a cleaved hinge region variant, it is impossible to draw definite conclusions; it is conceivable that partial, or less stable, insertion of the hinge region into the A sheet is still able to provoke and maintain the R form of the serpin. Indeed, Schulze et al. (1992) has shown that incubation of α_1-AT with peptides corresponding to only part of the reactive site loop and hinge region is able to provoke structural changes akin to those caused by cleavage of the reactive site loop.

There is now substantial evidence that serpins operate by the suicide substrate mechanism presented in Scheme 1 (Cooperman et al., 1993; Fish & Björk, 1979; Hood et al., 1994; Hopkins et al., 1993; Olson, 1985; Patston et al., 1991). The step leading to cleaved inhibitor (EI $\rightarrow$ E + I′) is irreversible because of the complete insertion of the reactive-site loop and hinge region as strand 4 in the A β-sheet, which in α_1-AT results in the separation of the P_1 and P'_1 residues by 67 Å (Löbermann et al., 1984). The step leading to the stable complex (EI $\leftrightarrow$ EI*) may be reversible (Shieh et al., 1989), but at a very low rate, k_{-2} being very slow; the value of k_4 is also small, such that the half-life of the serpin−enzyme complex is usually measured in days. The small values of k_{-2} and k_4 give rise to K_i values for serpin−enzyme inhibition reactions that are usually nanomolar or less. The mechanism of Scheme 1 represents a minimal reaction pathway for the interaction of serpins with proteases. Each of the steps of this mechanism could consist of more than one step. For instance, EI could be formed by an initial encounter complex which then isomerizes prior to the partitioning of the pathways. Such a step has been included by others in the reaction pathway (Cooperman et al., 1993; Hood et al., 1994; Patston et al., 1991). However, we have limited our description to serpin forms for which there is direct experimental evidence, i.e., initial and stable complexes and cleaved serpin. The ratio k_3/k_2 is known as the partition ratio, defined as r (Waley, 1985); r is related to the stoichiometry of inhibition (SI), which is the number of moles of inhibitor required to inhibit one mole of enzyme, by the relationship SI = 1 + r. The value of r may be affected in a number of instances by reaction conditions such as temperature (Hermans et al., 1995; Patston et al., 1991), or in the case of antithrombin with thrombin by ionic strength and heparin (Olson, 1985). Hinge region mutants of serpins appear to affect the partition of reaction products between the two pathways; i.e., they affect the ratio of k_3 to k_2 (Hood et al., 1994; Hopkins et al., 1993). For serpins, it has been proposed that the formation of the stable complex (EI*) from EI involves movement of the hinge region (Hood et al., 1994; Hopkins et al., 1993; Huber & Carrell, 1989; Potempa et al., 1994; Skriver et al., 1991); hinge region mutants are thus expected to affect the values of k_2, and therefore SI. The SI value of about 4 obtained from titration experiments for $P_{12}E$ is corroborated by SDS−PAGE and sequence analysis of the reaction products generated by the incubation of $P_{12}E$ with elastase which gave ~25% complexes and ~75% reactive-site loop cleaved inhibitor (Figure 4B), in keeping with the suicide substrate model.

The rate constant for the formation of the stable complex (k_{obs}) is the product of the apparent second-order rate constant for the formation of the stable complex (k_{ass}) and the concentration of serpin ([I]):

$$k_{obs} = k_{ass}[I] \quad (1)$$

The value for k_{ass} can be calculated either on the basis of the total concentration of serpin ($[I]_t$) or by using the so-called concentration of "active" serpin derived from the stoichiometry of inhibition (SI). The concentration of "active" serpin equals $[I]_t$/SI or $[I]_t/(1 + k_3/k_2)$. Waley (1985) has shown that the amount of cleaved inhibitor generated is equal to r.$[E]_t$; i.e., the amount of cleaved inhibitor will depend on the total concentration of enzyme ($[E]_t$) and the partition ratio ($r = k_3/k_2$). For the determination of k_{ass} in the present study, $[I]_t$ was always much greater than r.$[E]_t$; thus, depletion of the inhibitor due to the cleavage pathway

could be ignored, and the use of the total concentration of inhibitor for the calculation of k_{ass} is the correct method. The value of k_{ass} calculated using "active" serpin concentration in fact equals k_{ass}.SI. Table 2 gives this value for each of the variants of α_1-AT examined in the present study together other variant from previous studies. Interestingly, the value of k_{ass}.SI remains constant despite widely different values of SI. This situation occurs if the values of k_2 and k_3 are much greater that those of k_1.[I] and k_{-1}; i.e., if the first step is rate-limiting for the formation of the stable complex and cleaved inhibitor. Under these conditions, it can be shown that:

$$k_{ass} = k_1/(1 + k_3/k_2) = k_1/\mathrm{SI} \quad (2)$$

Thus, the k_{ass}.SI value will be constant irrespective of the value of SI and will equal k_1 when the first step is rate-limiting. Moreover, the data obtained in the present and previous studies (Table 2) are consistent with the first step being rate limiting in general, since k_{ass}.SI did not vary for a number of mutants with several proteases. It should be noted that the reaction mechanism proposed by Hood et al. (1994) includes an additional step before the partitioning of the pathway between the inhibition and cleavage reactions. For this scheme, the rate-limiting step could be either the initial association step or the additional step before the partitioning.

The results obtained for the P_{10}E mutation reveal a new aspect of the involvement of hinge region residues in the serpin reaction mechanism. The P_{10}E mutation appears to have affected the stability of the complex rather than the rate of insertion of the hinge region into the A-sheet. P_{10}E dissociated slowly and irreversibly from elastase and was found to be cleaved at the $P_1-P'_1$ bond. This release could result from an increase in k_{-2}, leading to an increase in the equilibrium concentration of EI which will favor the formation of E + I'. In other words, since EI and EI* are in equilibrium, there will always be a slow production of I' from EI*, and increasing the rate of the slowest step (k_{-2}) will increase the rate of formation of cleaved inhibitor. Alternatively, the release of cleaved inhibitor could occur not through a reversal of the inhibitory pathway but through an extension of it (k_4) (Cooperman et al., 1993; Patston et al., 1991); the P_{10}E mutation may have increased this rate.

Data presented in this work and the pattern of residue conservation in the hinge region do not fit the hypothesis that conservation of the hinge region sequence is due solely to a requirement for small side-chains to enable rapid insertion into the A-sheet, which is in turn required for the formation of a stable complex (Huber & Carrell, 1989; Potempa et al., 1994; Skriver et al., 1991). The conserved sequence (Table 1) includes residues whose side-chains would be solvent exposed; mutation of these residues to the nonconservative glutamate had no effect on inhibition. Mutation of the P_8 residue whose side-chain is buried in the structure of cleaved α_1-AT (Löbermann et al., 1984) also had no effect on inhibition. Nevertheless, the degree of conservation of these residues is not significantly less than that of residues whose mutation did affect inhibition. Thus, although much experimental evidence suggests that insertion of the hinge region of serpins into the A-sheet occurs during inhibition, conservation of residues whose side-chains remain solvent-exposed suggests that the hinge region may have more than one role to play.

Serpins are capable of many conformations, each being quite stable. The first of these is the serpin prior to contact with the enzyme, which probably has a five-stranded β-sheet A and a helical reactive-site loop as seen in the ovalbumin crystal structure (Stein et al., 1990) or the α_1-antichymotrypsin crystal structure (Wei et al., 1994). The second structure is the well-known structure of the reactive-site loop-cleaved serpin with the hinge region and reactive-site loop incorporated into the A-sheet (Baumann et al., 1991; Delarue et al., 1990; Löbermann et al., 1984). Plasminogen activator inhibitor-1 and antithrombin show a third structure (Carrell et al., 1994; Mottonen et al., 1992), where dissociation of strand 1C from the C-sheet has permitted the reactive-site loop and hinge region to be fully incorporated into the A-sheet without cleavage of the reactive-site loop. A fourth structure is that of the partially inserted hinge region of antithrombin (Carrell et al., 1994; Schreuder et al., 1994). In addition to these crystal structures, a number of alternative serpin conformations have been identified or inferred. The most important of these is the structure of the serpin in complex with the enzyme. It is believed that in this structure the hinge region is at least partially embedded in the A-sheet; it must therefore make a turn to emerge from this sheet. Polymerized serpins (Lomas et al., 1992; Mast et al., 1992) and a substrate form of plasminogen activator inhibitor-1 (Declerck et al., 1992) are other serpin structural variants. All forms for which the crystal structures are available differ primarily in the arrangement of the hinge region and reactive-site loop. The serpin hinge region structure also probably varies as a part of the normal inhibition process, moving from a solvent-exposed strand to partial incorporation into a β-sheet, and the rate of this transformation appears to be significant for effective inhibitor function. Thus, it would appear that versatility is a prime requirement for hinge region residues, allowing the formation of a number of different structures. Alanines, apart from being common in helices, are also suited to straplike connections, such as Greek keys, β-arches or $\alpha-\beta$ connections, and are fairly indifferent to solvent exposure (Richardson & Richardson, 1989). Alanines are therefore very versatile and appear to suit the requirement for the hinge region motif. In addition to allowing the existence of alternative reactive-site loop and hinge region structures, the hinge region could also conceivably be significant to the folding of serpins. The hinge region has long been predicted to form an α-helix because of its high alanine content (Garnier et al., 1978; Löbermann et al., 1984). It seems possible that early in the folding pathway, the hinge region does indeed adopt a helical structure, and that this prevents it from participating in the formation of β-sheet A, allowing the folding of the inhibitory form of the serpin. A comprehensive explanation for the conservation of hinge region residues in the inhibitor members of the serpin family thus still eludes us, perhaps owing to the multitude of roles that it plays.

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