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The P$_6$−P$_2$ Region of Serpins Is Critical for Proteinase Inhibition and Complex Stability[†]

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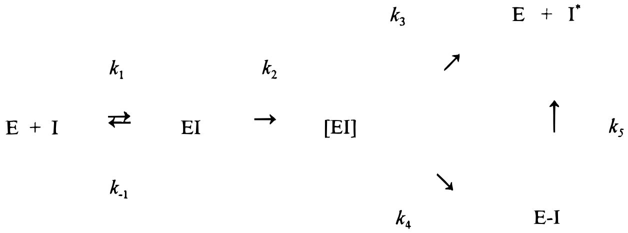
ABSTRACT: Two of the prototypic serpins are α_1-proteinase inhibitor and ovalbumin. α_1-Proteinase inhibitor is a rapid inhibitor of a number of proteinases and undergoes the characteristic serpin conformational change on cleavage within the reactive center loop, whereas ovalbumin is noninhibitory and does not undergo the conformational change. To investigate if residues from P$_{12}$ to P$_2$ in the reactive center loop of ovalbumin are intrinsically incapable of being in an inhibitory serpin, we have made chimeric α_1-proteinase inhibitor variants containing residues P$_{12}$−P$_7$, P$_6$−P$_2$, or P$_{12}$−P$_2$ of ovalbumin and determined their inhibitory properties with trypsin and human neutrophil elastase. With the P$_{12}$−P$_7$ and P$_6$−P$_2$ variants, the steps before and after the fork of the branched suicide-substrate pathway were affected as reflected by changes in rates and stoichiometries of inhibition with both proteinases. The P$_{12}$−P$_2$ variant showed that those effects were nonadditive, with exclusive substrate behavior for elastase and only residual inhibitory activity against trypsin. The properties of the variants were consistent with them obeying the suicide-substrate mechanism characteristic of serpins. Enzyme activity was regenerated from complexes formed with the P$_6$−P$_2$ variant faster than with wild-type indicating that the rate of turnover of the complex was increased. Based on proteinase susceptibility in the reactive center loops of the P$_6$−P$_2$ and P$_{12}$−P$_2$ variants, and on an increase in heat stability of the cleaved P$_{12}$−P$_2$ variant, it was concluded that the variants had undergone complete loop insertion on cleavage. These results show that the reactive center loop residues P$_{12}$−P$_2$ of ovalbumin can be present in inhibitory serpins although decreasing the inhibitory properties. These data also demonstrate that the residues in the P$_6$−P$_2$ region of serpins are critical for rapid inhibition of proteinases and formation of stable serpin−proteinase complexes.

The serpins are a family of structurally similar proteins, many of which are inhibitors of serine proteinases. They regulate a wide variety of physiological processes including blood coagulation, fibrinolysis, and inflammation (Gettins et al., 1996). Proteinase inhibition occurs through formation of a covalent, bimolecular serpin−proteinase complex. The complex is believed to be trapped at the acyl-enzyme phase of the catalytic mechanism (Lawrence et al., 1995; Plotnick et al., 1996). The mechanisms which stabilize this complex and prevent deacylation are not understood; however, the serpin must undergo a major conformational change during this process (Shore et al., 1995; Wilczynska et al., 1995; Strömqvist et al., 1996; Stratikos & Gettins, 1997). The reaction is best described by a suicide-substrate mechanism (Rubin et al., 1990; Björk et al., 1992; Patston et al., 1994; Hood et al., 1994; Wright & Scarsdale, 1995), as shown in Scheme 1.

Scheme 1



A critical feature of this scheme is the formation of an intermediate proteinase−serpin complex, [EI], in which the proteinase active site has reacted with the serpin P$_1$−P$_{1'}$ peptide bond.[1] This intermediate can react to form the stable covalent complex, E−I, or the proteinase can cleave the serpin as in a normal substrate reaction to give free enzyme and serpin cleaved in the reactive center at the P$_1$−P$_{1'}$ bond (I*). In this scheme the rate of insertion of the reactive center loop into β-sheet A is a critical determinant of the outcome of the reaction. If this rate is faster than the rate of deacylation of [EI] then k_4 will predominate, but if it is slower then k_3 will predominate. The stable complex turns over very slowly to produce free enzyme and reactive center-cleaved serpin (k_5). Although serpins meet the criteria for suicide substrates (chemically unreactive in the absence of enzyme, activated specifically by its target enzyme, and once activated k_4 is greater than k_3), it has been suggested that modifications of Scheme 1 might be needed to explain all

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[1] Using the nomenclature of Schechter and Berger (1967), the P$_1$−P$_{1'}$ peptide bond is that which is cleaved by the proteinase. Residues N-terminal to this bond are designated P$_2$, P$_3$, and so on, and those C-terminal are designated P$_{2'}$, P$_{3'}$, and so on.

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the properties of serpins (Stone & Le Bonniec, 1997; O'Malley et al., 1997).

Two of the best studied members of the serpin family are α_1-proteinase inhibitor and ovalbumin. α_1-Proteinase inhibitor (also called α_1-antitrypsin) is a rapid inhibitor of neutrophil elastase. Deficiency of this serpin can lead to emphysema as a result of the unregulated action of elastase on the lung tissue (Eriksson, 1996). Ovalbumin shares about 30% sequence homology with α_1-proteinase inhibitor (Hunt & Dayhoff, 1980) and has a very similar three-dimensional structure (Stein et al., 1991; Elliott et al., 1996), but it is not an inhibitor of serine proteinases (Wright, 1984). The reason for ovalbumin being a noninhibitory serpin has been believed to be because the P_{14} arginine residue cannot insert into β-sheet A (Wright et al., 1990), thereby preventing the serpin from undergoing the conformational change on interaction with a proteinase (Gettins, 1989). However, there is evidence that uncleaved ovalbumin can undergo partial loop insertion, from residues P_{14} to P_{10}, to give the more stable S-ovalbumin (Huntington et al., 1995). Although variants of inhibitory serpins with mutations at or near P_{14} are predominantly substrates for their target proteinases, in many cases they retain some inhibitory activity and can loop insert (Hood et al., 1994; Wright & Scarsdale, 1995; Aertgeerts et al., 1995; Lukacs et al., 1996; Gils et al., 1996). In addition, P_{14} Arg to Ser mutants of ovalbumin can loop insert on cleavage but still remain noninhibitory (Huntington et al., 1997). Thus, the lack of inhibitory properties of ovalbumin cannot be explained in terms of an inappropriate hinge region sequence only.

We tested if the reactive center loop of ovalbumin from residues P_{12} to P_2 can be present in inhibitory serpins by creating α_1-proteinase inhibitor (α_1-PI)[2] variants containing residues $P_{12}-P_7$, P_6-P_2, or $P_{12}-P_2$ of ovalbumin. We determined the rates and stoichiometries of inhibition of trypsin and human neutrophil elastase (HNE) and the extent of loop insertion. Comparison of the properties of the three variants shows that residues $P_{12}-P_2$ of ovalbumin can fully insert into β-sheet A of α_1-PI. However, the $P_{12}-P_7$ and P_6-P_2 sequences affect steps both before and after the fork of the branched pathway. The $P_{12}-P_2$ variant showed that those effects were cooperative, leading to the complete loss of inhibitory properties in the case of HNE. Reactivation of trypsin from complexes formed with variants containing the P_6-P_2 mutation showed that this region affects the value of k_5. These results demonstrate for the first time that residues in the P_6-P_2 region are critical not only for rapid interaction with the proteinase but also for the stabilization of the serpin−proteinase complex.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from Pharmacia (Piscataway, NJ) and oligonucleotides synthesized by DNA Agency (Malvern, PA). Sequenase was from United States Biochemical (Cleveland, OH). T4 ligase was from New England Biolabs Inc. (Beverly, MA). pET16b vector was from Novagen (Madison, WI). HNE was from Athens Research (Athens, GA). β-Trypsin was kindly provided by Efstratios Stratikos (Department of Biochemistry, University of Illinois at Chicago). Methoxysuccinyl-(Ala)$_2$-Pro-Val-*p*-nitroanilide and PMSF were from Sigma (St. Louis, MO). S-2222 was from Chromogenix (Franklin, OH). Papain was from Boehringer (Indianapolis, IN).

	P14	P13	P12	P11	P10	P9	P8	P7	P6	P5	P4	P3	P2	P1	P1'
α1-PI	T	E	A	A	G	A	M	F	L	E	A	I	P	M*	S
Ovalbumin	R	E	V	V	G	S	A	E	A	G	V	D	A	A	S
P_{12}-P_7OV-α1-PI	T	E	*V*	*V*	*G*	*S*	*A*	*E*	L	E	A	I	P	M*	S
P_6-P_2OV-α1-PI	T	E	A	A	G	A	M	F	*A*	*G*	*V**	*D*	*A*	M*	S
P_{12}-P_2OV-α1-PI	T	E	*V*	*V*	*G*	*S*	*A*	*E*	*A*	*G*	*V*	*D*	*A*	M*	S

FIGURE 1: Amino acid sequences in the reactive center loop of α_1-PI and ovalbumin and in the α_1-PI reactive center loop chimeras. The P nomenclature used is that of Schechter and Berger, 1967. The residues substituted in the α_1-PI reactive center loop chimeras are underlined. The asterisk (*) indicates the HNE cleavage sites deduced from amino-terminal sequence analysis of the proteolysis mixture under conditions described in Materials and Methods.

Mutagenesis of Wild-Type and Reactive Center Loop Chimera Variants of α_1-Proteinase Inhibitor. The full length wild-type α_1-PI cDNA was contained in a *Hin*dIII-*Xba*I fragment in the pMAStop vector (Hood et al., 1994). Site-directed mutagenesis was performed using PCR-based techniques. In order to get optimal expression of a soluble, fully active recombinant α_1-PI, we deleted the first five codons (Johansen et al., 1987) by introducing a new *Nco*I site as the initiation site. The amplified *Nco*I-*Xba*I fragment encoding the newly constructed wild-type α_1-PI DNA was then subcloned into the pET16b vector. The reactive center loop chimera mutants were constructed on this wild-type background using oligonucleotides encoding for the sequence of ovalbumin. Regions amplified by PCR were sequenced to confirm the existence of the desired mutation together with the correct initiation and stop sites. The amino acid sequences of the reactive center loop of α_1-PI, ovalbumin, and the mutants are shown in Figure 1. The mutants made contained residues $P_{12}-P_7$, P_6-P_2, or $P_{12}-P_2$ of ovalbumin and were designated ($P_{12}P_7$OV)-α_1-PI, (P_6P_2OV)-α_1-PI, or ($P_{12}P_2$OV)-α_1-PI, respectively.

Expression, Refolding, and Purification of Recombinant α_1-Proteinase Inhibitor. The recombinant proteins (wild-type and mutants) were produced as inclusion bodies in *Escherichia coli*. The recovery of the active proteins from the inclusion bodies was done as previously described (Kwon et al., 1995) with slight modifications. Briefly, the *E. coli* BL21 (DE3) cells were grown at 37 °C on LB medium containing 125 μg/mL ampicillin. When A_{600} reached 0.8, IPTG was added to a final concentration of 1 mM, and growth was prolonged for 3 h. We found that the recombinant α_1-PI proteins accumulated mainly as an inactive form in inclusion bodies, either when growth was performed at 30 or 37 °C or when different times of induction were used. Cells from a 1 L culture were resuspended in buffer A (0.1 M Tris-HCl, pH 8.1, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF) and disrupted by sonication (30 s × 4). The collected pellet (12000g for 10 min) of inclusion bodies was washed twice in buffer A containing 0.5% Triton X-100 and solubilized in 8 M urea in buffer A at a protein concentration of 0.4 mg/mL with the aid of sonication. The protein solution was diluted 5-fold with buffer B (10 mM phosphate buffer, pH 6.5, 1 mM EDTA, 1

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[2] Abbreviations: α_1-PI, α_1-proteinase inhibitor; SI, stoichiometry of inhibition; ($P_{12}P_7$OV)-α_1-PI, α_1-proteinase inhibitor containing residues $P_{12}-P_7$ of ovalbumin; (P_6P_2OV)-α_1-PI, α_1-proteinase inhibitor containing residues P_6-P_2 of ovalbumin; ($P_{12}P_2$OV)-α_1-PI, α_1-proteinase inhibitor containing residues $P_{12}-P_2$ of ovalbumin; HNE, human neutrophil elastase; CD, circular dichroism.

mM β-mercaptoethanol) and dialyzed against buffer B. The dialysate was loaded onto a DE-52 column (2.5 × 6 cm) equilibrated with buffer B, and proteins were eluted with a linear gradient of 0−0.2 M NaCl in buffer B. Purity and oligomerization state of the proteins were assessed by SDS and nondenaturing polyacrylamide gel analysis. Fractions containing pure monomeric forms of recombinant α_1-PI were pooled and concentrated, and buffer was exchanged with 0.1 M Tris-HCl, pH 8.1, and 1 mM β-mercaptoethanol by ultrafiltration on an Amicon PM10 membrane. Protein concentrations were determined spectrophotometrically using the extinction coefficient of the plasma protein, $A_{280\mathrm{nm}}{}^{1\%} = 5.3$ (Pannell et al., 1974), and a molecular weight of 44 000 for the recombinant proteins (Kwon et al., 1995).

Determination of the Stoichiometry of Inhibition. Stoichiometry of inhibition (SI) values for the inhibition of trypsin were determined by incubating different concentrations of wild-type and ($P_{12}P_7$OV)-α_1-PI for 30 min at 25 °C, with 3 nM of trypsin in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 0.1 mM EDTA, and 0.1% PEG 8000. The residual amidolytic activity was determined by the addition of 100 μM S-2222. Linear regression analysis of the decrease in proteinase activity with increasing concentration of α_1-PI yielded the estimates for the stoichiometry of inhibition as the intercept on the abscissa. The SI for trypsin and (P_6P_2-OV)-α_1-PI was determined in the same way except an incubation time of 6 h was used due to the slower rate of inhibition. It was not possible to incubate to completion due to the reactivation of trypsin activity at longer time points (see Results and Discussion); consequently, the SI value is only approximate. With ($P_{12}P_2$OV)-α_1-PI and trypsin the reactivation was even more rapid and an incubation time of 1 h was used. SI values for the inhibition of HNE were determined by incubating different concentrations of wild-type and ($P_{12}P_7$OV)-α_1-PI for 10 min at 25 °C, with 5 nM HNE in 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000. The residual amidolytic activity was determined by the addition of 200 μM methoxysuccinyl-(Ala)$_2$-Pro-Val-*p*-nitroanilide. With (P_6P_2OV)-α_1-PI an incubation time of 1.5 h was used. In this case reactivation of HNE activity was apparent at longer times; hence, it was impossible to incubate the reaction to completion, and the value determined for the SI is approximate.

Rates of Inhibition of Trypsin and Elastase by Wild-Type and α_1-PI Variants. The rate of inhibition of trypsin by recombinant α_1-PI was determined at 25 °C by use of a discontinuous assay procedure. Under pseudo-first-order conditions, 150 nM wild-type α_1-PI or ($P_{12}P_7$OV)-α_1-PI or 5 μM (P_6−P_2OV)-α_1-PI was incubated with 12.5 nM trypsin in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 10 mM CaCl$_2$, and 0.1% PEG 8000. With ($P_{12}P_2$OV)-α_1-PI the concentration was 20 μM and trypsin was 6.25 nM, in the same buffer. The residual trypsin activity was determined at various times by diluting the reaction mixture into the assay buffer containing 100 μM S-2222. The pseudo-first-order constant, k_{obs}, for the reaction was obtained from the slope of a semilog plot of the residual trypsin activity against time, and the second-order rate constant, k_{app}, was determined by $k_{\mathrm{obs}}/[\mathrm{I}_0]$, where $[\mathrm{I}_0]$ is the initial serpin concentration.

For the wild-type α_1-PI and ($P_{12}P_7$OV)-α_1-PI, the rate of HNE inhibition was measured by use of a continuous assay procedure; 0.5 nM HNE was incubated with 2.5 and 5 nM of wild-type α_1-PI or ($P_{12}P_7$OV)-α_1-PI in the presence of 200 μM methoxysuccinyl-(Ala)$_2$-Pro-Val-*p*-nitroanilide. The reaction was continuously recorded at 405 nm. The rate constant for the reaction was obtained after fitting the reaction curve to the equation $k_{\mathrm{app}} = (k_{\mathrm{ass}} \cdot I_0)/(1 + [S_0/K_{\mathrm{M}}])$, where K_{M} is the value for HNE with the substrate.

The rate of inhibition of HNE by (P_6P_2OV)-α_1-PI and ($P_{12}P_2$OV)-α_1-PI was assessed using the discontinuous assay; 2.3 μM (P_6P_2-OV)-α_1-PI or 10 μM ($P_{12}P_2$OV)-α_1-PI was incubated with 50 nM HNE in 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000. The residual HNE activity was determined at various times by diluting the reaction mixture into the assay buffer containing 100 μM methoxysuccinyl-(Ala)$_2$-Pro-Val-*p*-nitroanilide. In the case of ($P_{12}P_2$OV)-α_1-PI no inhibition was measured. For (P_6P_2OV)-α_1-PI the SI for this reaction was about 60; consequently, the "inhibitory" concentration of (P_6P_2OV)-α_1-PI was taken to be 38.3 nM. The data were analyzed using the second-order rate equation.

The rate constant k_{app} determined by both the discontinuous and continuous assays is defined as $k_{\mathrm{app}} = (k_2/K_{\mathrm{s}})[k_4/(k_4 + k_3)]$. This term is dependent on the amount of partitioning along the substrate, noninhibitory pathway as indicated by the $k_4/(k_4 + k_3)$ term. The SI is defined as $(k_4 + k_3)/k_4$; therefore, multiplying k_{app} by the SI will give the rate constant k'_{app}, which represents the formation of [EI] (Hood et al., 1994; Gettins et al., 1996). If the proteinase also cleaves within the reactive center at a site other than the P_1−$P_{1'}$ bond, this analysis cannot be used as it is not possible to separate this inactivation of the serpin from the inactivation by cleavage at the P_1−$P_{1'}$ bond (k_3).

Stability of Complexes between Trypsin and α_1-PI Variants. Trypsin (12.5 nM) was incubated in the presence of 25 nM wild-type α_1-PI and ($P_{12}P_7$OV)-α_1-PI, 50 nM (P_6P_2-OV)-α_1-PI, or 250 nM ($P_{12}P_2$OV)-α_1-PI in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 10 mM CaCl$_2$, and 0.1% PEG 8000 at 25 °C. At different times, the residual trypsin activity was measured by dilution of an aliquot of the incubated material into buffer containing 100 μM substrate S-2222. To determine if dissociation of the complex could be enhanced by addition of a nucleophile (at 1 M concentration), the same procedure was carried out except, after 4 h of incubation of trypsin with (P_6P_2OV)-α_1-PI, an equal volume of either buffer, 2 M ammonium hydrochloride, or 2 M methylamine hydrochloride (both in the incubation buffer at pH 7.4) was added to the incubation mixture. The sample was then incubated further at 25 or 37 °C and assayed for trypsin activity. To determine if a higher pH was required to see an effect of the nucleophile, some experiments were carried out with the buffer at pH 8.0.

Reactive Center Loop Cleavage of Wild-Type and Variant α_1-PI. Papain was used to cleave wild-type α_1-PI (1/26 ratio) in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl for 20 min at 37 °C (Mast et al., 1992). The reaction was stopped by 10 mM iodoacetamide. The cleaved protein lacks the hexapeptide Leu^{352}−Met^{358} (P_6−P_1) due to double cleavage at P_7−P_6 and P_1−$P_{1'}$ (Mast et al., 1992). For all of the α_1-PI variants, cleavage was done using HNE and characterization of the cleavage site was performed by N-terminal sequence analysis of the cleavage reaction mixture after stopping the reaction by addition of PMSF to 5 mM and freezing until analysis. Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer, by the protein sequencing facility in the Department of Biochemistry, University of Illinois-Chicago. ($P_{12}P_7$OV)-α_1-PI and wild-type α_1-PI were cleaved by HNE (1/5 ratio) in 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000 for 10 min at 25 °C. (P$_{12}$P$_2$OV)-α_1-PI was cleaved by HNE (1/100 ratio) in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 10 mM CaCl$_2$, and 0.1% PEG 8000 for 30 min at 25 °C. (P$_6$P$_2$-OV)-α_1-PI was cleaved by HNE (1/88 ratio) in 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000 for 20 min at 37 °C. Completion of the cleavage reactions for (P$_6$P$_2$OV)-α_1-PI and (P$_{12}$P$_2$OV)-α_1-PI was demonstrated by SDS−gel electrophoresis.

Extent and Rate of Reactive Center Loop Insertion: Kinetics of Cleavage of (P$_6$P$_2$OV)-α_1-PI and (P$_{12}$P$_2$OV)-α_1-PI by HNE. For this aim we took advantage of the fact that the (P$_6$P$_2$OV)-α_1-PI variant was cleaved by HNE (see above and the results section) at two sites within the reactive center loop: at P$_4$−P$_3$ and at P$_1$−P$_{1'}$ as deduced from the sequence analysis performed on the proteolysis mixture. These two single cleaved products could be resolved by separation on Mono-Q HR5/5 using a linear gradient from 5 to 150 mM phosphate buffer, pH 7.0. N-Terminal sequence analysis was performed again on each purified material to determine the corresponding cleavage site. The time course of cleavage of the (P$_6$P$_2$OV)-α_1-PI variant was followed by the Mono-Q elution profiles of reactions of 5.8 μM of the variant with 66 nM HNE at 37 °C in buffer consisting of 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000. At different times, the reactions were stopped by addition of PMSF to 5 mM and microcentrifuged for 5 min in order to remove any precipitated insoluble PMSF. The recovered supernatant was diluted 10 times in the low ionic strength Mono-Q buffer, applied to the column, and eluted using the same gradient as described above. Proteins were detected using a Shimadzu RF535 fluorescence detector (with $\lambda_{\mathrm{ex}} = 280$ nm and $\lambda_{\mathrm{em}} = 340$ nm) and quantified according to their elution peak fluorescence intensity area expressed in millivolts × seconds after analysis using the program Millenium from Millipore. Peak areas were normalized to the total species, cleaved and uncleaved, present. The resulting fractions of each cleavage product of the (P$_6$P$_2$OV)-α_1-PI variant were fitted, using Scientist for Windows, to a model for parallel pseudo-first-order reaction (that is, reactant X going to product A or B).

Further cleavage of the two single cleaved (P$_6$P$_2$OV)-α_1-PI species was achieved by letting the reaction continue for 20 h. After Mono-Q separation the two newly cleaved products were analyzed by N-terminal sequencing to determine if the new cleavage site was within the reactive center loop. The same approach was followed with the (P$_{12}$P$_2$OV)-α_1-PI variant, although only the P$_1$−P$_{1'}$ bond cleavage site was initially observed.

Circular Dichroism Spectroscopy. CD spectra were recorded on a JASCO 710 spectropolarimeter. The effect of temperature on changes in protein secondary structure were followed by recording the ellipticity at 222 nm every 0.5 °C in a 0.1 cm temperature-controlled cell. A change of 30 °C/h was applied using a Neslab RTE-110 water bath. Before each temperature scan, a spectrum in the range of 200−260 nm was recorded from which a spectrum of the reference blank was subtracted and then smoothed. Protein samples (0.3 mg/mL in 20 mM sodium phosphate, pH 7.6) were microfuged and degassed prior to use.

Table 1: SI Values and Apparent Second-Order Rate Constants (k_{app}) for Inhibition of Trypsin and HNE by α_1-PI and α_1-PI Reactive Center Loop Chimeras[a]

proteinase	α1-PI variant	SI	k_{app} ($\mathrm{M}^{-1}\,\mathrm{s}^{-1}$)
trypsin	wild-type	1.1	1.69×10^5
	(P$_{12}$P$_7$OV)-α1-PI	1.1	5.79×10^4
	(P$_6$P$_2$OV)-α1-PI	∼6[b]	0.99×10^3
	(P$_{12}$P$_2$OV)-α1-PI	∼100[b]	1.27×10^2
HNE	wild-type	1.2	9.00×10^6
	(P$_{12}$P$_7$OV)-α1-PI	1.5	4.34×10^6
	(P$_6$P$_2$OV)-α1-PI	∼60[b]	3.28×10^4
	(P$_{12}$P$_2$OV)-α1-PI	nd	nd

[a] Conditions of assays are as described under Materials and Methods. [b] For these variants, complete inhibition was never achieved and the value given is an estimate of the SI determined at the time of maximum inhibition. nd, not determined due to no measurable inhibition.

RESULTS AND DISCUSSION

Reaction of Chimeric α_1-PI Variants with Trypsin and Elastase. In this study we have investigated the hypothesis that the reactive center loop of ovalbumin is intrinsically inimical to being in an inhibitory serpin by creating chimeric α_1-PI molecules containing residues P$_{12}$−P$_7$, P$_6$−P$_2$, or P$_{12}$−P$_2$ of ovalbumin. By keeping the P$_1$−P$_{1'}$ bond as Met-Ser and the P$_{14}$ as threonine, the chimeric serpins should still be recognized by trypsin and HNE and be able to loop insert at P$_{14}$. The α_1-PI variants were tested for their ability to inhibit trypsin and HNE by measuring the inhibition rate constants and the stoichiometries of inhibition (Table 1) and for their ability to form covalent complexes with the proteinases stable under SDS−PAGE conditions (Figure 2). The (P$_{12}$P$_7$OV)-α_1-PI variant had a slightly reduced rate of trypsin inhibition (k_{app}), compared to wild-type, with no apparent change in SI. The SI is determined by $(k_3 + k_4)/k_4$, and therefore if k_4 is very large relative to k_3 even big changes in the two rate constants (relative to wild-type) might not be detectable. Consequently with (P$_{12}$P$_7$OV)-α_1-PI the rate of loop insertion might be decreased but without a detectable effect on the SI. This is probably the case since there is a small increase in the SI for the inhibition of HNE by (P$_{12}$P$_7$OV)-α_1-PI compared to wild-type. This is consistent with previous data showing the dependence of the SI on the proteinase (Hood et al., 1994). The rate of inhibition of HNE was also slightly reduced with the (P$_{12}$P$_7$OV)-α_1-PI variant. The site of cleavage by HNE in the reactive center loop of (P$_{12}$P$_7$OV)-α_1-PI was found to be the P$_1$−P$_{1'}$ bond (see below); therefore, the change in SI was not due to cleavage elsewhere in the reactive center loop. In this case it is possible to multiply SI by k_{app} to give the parameter k'_{app}, the rate constant for the formation of [EI]. For the inhibition of HNE by wild-type α_1-PI, k'_{app} is $1.08 \times 10^7\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$, and for inhibition of HNE by (P$_{12}$P$_7$OV)-α_1-PI, k'_{app} is $6.51 \times 10^6\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$. This reduction in k'_{app} shows that the P$_{12}$−P$_7$ residues affect the steps *prior to* the branch point, that is, K_{s} and k_2. This was an unexpected finding as previously these residues were presumed only to influence loop insertion and SI. This shows that the P$_{12}$−P$_7$ residues can influence the conformation around the P$_1$−P$_{1'}$ bond and hence the interaction with the proteinase.

The inhibitory properties of (P$_6$P$_2$OV)-α_1-PI were significantly changed compared to wild-type α_1-PI. For the inhibition of trypsin and HNE the k_{app} values were reduced by 170- and 270-fold, respectively, compared to wild-type

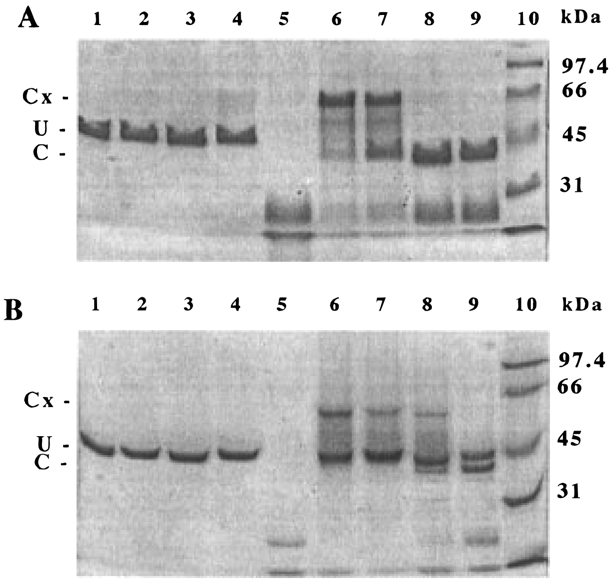

FIGURE 2: (A) Reaction of α_1-PI reactive center loop variants with HNE: lanes 1−4, 2 μg (1.5 μM) of wild-type α_1-PI, ($P_{12}P_7$OV)-α_1-PI, (P_6P_2OV)-α_1-PI, and ($P_{12}P_2$OV)-α_1-PI; lane 5, 2.5 μg (2.8 μM) of HNE; lanes 6−9, 4 μg (3 μM) of wild-type α_1-PI, ($P_{12}P_7$-OV)-α_1-PI, (P_6P_2OV)-α_1-PI, and ($P_{12}P_2$OV)-α_1-PI incubated for 10 min at 25 °C with 1.25 μg (1.4 μM) of HNE in Hepes (0.1 M, pH 8.0), NaCl (0.5 M), and PEG 8000 (0.1%); lane 10, molecular weight markers. Cx stands for complex, C for cleaved, and U for uncleaved. After incubation, the samples were boiled for 5 min and run on a 12% SDS−polyacrylamide gel under reducing conditions. (B) Reaction of α_1-PI reactive center loop variants with trypsin: lanes 1−4, 2 μg (1.5 μM) of wild-type α_1-PI, ($P_{12}P_7$OV)-α_1-PI, (P_6P_2OV)-α_1-PI, and ($P_{12}P_2$OV)-α_1-PI; lane 5, 2 μg (3 μM) of trypsin; lanes 6−9, 4 μg (3 μM) of wild-type α_1-PI, ($P_{12}P_7$OV)-α_1-PI, (P_6P_2OV)-α_1-PI, and ($P_{12}P_2$OV)-α_1-PI incubated for 20 min at 25 °C with 1 μg (1.5 μM) of trypsin in Hepes (0.1 M, pH 7.4), NaCl (0.1 M), $CaCl_2$ (10 mM), and PEG 8000 (0.1%); lane 10, molecular weight markers.

α_1-PI. The SI values for the inhibition of trypsin and HNE were about 6 and 60, respectively. It it generally believed that the hinge region residues (P_{14}−P_{10}) are the critical determinants of the rate of loop insertion and, hence, SI. The importance of residues close to the P_1−$P_{1'}$ bond in determining SI has not previously been considered, although this is reasonable assuming that full insertion is necessary for inhibition.

The most dramatic effects were seen with ($P_{12}P_2$OV)-α_1-PI. This mutant had only residual inhibitory activity against trypsin with an SI of about 100. No inhibition of HNE was detected, suggesting that in this case the serpin was behaving as a substrate with k_3 being so much greater than k_4 that no inhibition could be measured. To confirm this, the reactions involving the α_1-PI variants with trypsin or HNE were investigated by SDS−PAGE (Figure 2). With HNE (panel A) and trypsin (panel B) complexes were formed with wild-type and ($P_{12}P_7$OV)-α_1-PI, along with a small amount of cleaved serpin consistent with the rate constants and SIs for these reactions. With (P_6P_2OV)-α_1-PI no complex was detected with HNE even though some inhibition can be measured (Table 1). With trypsin there was some complex visible, consistent with the lower SI for this reaction. For ($P_{12}P_2$OV)-α_1-PI no complex was visible with either enzyme.

To assess the influence of the P_{12}−P_7 residues on the P_6−P_2 residues, and vice versa, we analyzed the data for k_{app} in terms of a double-mutant cycle for the inhibition of trypsin (Figure 3). Such an analysis was previously done to investigate the effects of mutations at P_2 and $P_{1'}$ in α_1-PI-Met^{358} (Hopkins et al., 1995). In this analysis the ratios of k_{app} for pairs of serpins are compared. Thus, the ratio WT/$P_{12}P_7$ is 2.9 and the ratio P_6P_2/$P_{12}P_2$ is 7.8. This shows that changing the P_{12}−P_7 residues has a greater effect when the P_6−P_2 are those of ovalbumin. Similarly, the ratio WT/P_6P_2 is 170 and the ratio $P_{12}P_7$/$P_{12}P_2$ is 456. In this case changing the P_6−P_2 residues has a greater effect when the P_{12}−P_7 residues are those of ovalbumin. This analysis unambiguously demonstrates that the effect of mutation in one half of the reactive center loop depends on the nature of the residues present in the other half. This indicates that nonadditive, or cooperative, interactions take place between the two halves of the reactive center loop. This could be because of changes in the native conformation of the reactive center loop as well as the conformations the loop adopts during the proteolytic cycle.

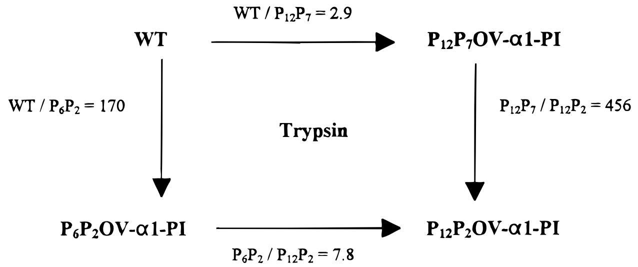

FIGURE 3: Nonadditive effects of changes within the reactive center loop of α_1-PI. The expression on top of each arrow represents the ratio of k_{app} for the inhibition of trypsin for the specified mutants; i.e., WT/P_6P_2 corresponds to k_{app} (wt α_1-PI)/k_{app}[(P_6P_2OV)-α_1-PI] with the values for k_{app} taken from Table 1. The effects of the P_{12}−P_7 and P_6−P_2 ovalbumin residues substituted in α_1-PI on the rate of inhibition of trypsin are not independent of each other as reflected by the difference in the magnitude of the decrease in rate of inhibition on parallel sides of the cycle; i.e., 2.9 versus 7.8 and 170 versus 456. Note that the product of each cycle is equal; i.e., $2.9 \times 456 = 1322$, compared to $170 \times 7.8 = 1326$. These values are equal to that for the complete mutation of wild-type α_1-PI to ($P_{12}P_2$OV)-α_1-PI; i.e., k_{app}(wt α_1-PI)/k_{app}[($P_{12}P_2$OV)-α_1-PI] = 1330.

During the course of these experiments it became clear that the complexes formed with (P_6P_2OV)-α_1-PI were not stable and that regeneration of active proteinase occurred. Thus, reactivation of the complexes formed with trypsin was studied (Figure 4). Complexes formed using wild-type α_1-PI or ($P_{12}P_7$OV)-α_1-PI showed no reactivation of trypsin activity during the course of the experiment. The (P_6P_2OV)-α_1-PI variant showed initial inhibition at a very slow rate followed by regeneration of trypsin activity. ($P_{12}P_2$OV)-α_1-PI showed only a transient inhibition of trypsin, and the complex that resulted from the inhibition was so unstable that complete regeneration of trypsin activity occurred rapidly. Because of this reactivation the values for SI reported in Table 1 for (P_6P_2OV)-α_1-PI and ($P_{12}P_2$OV)-α_1-PI are "best-estimate" values. The true SI, $(k_3 + k_4)/k_4$, is probably lower, but it is not possible to measure it due to the competing process of complex dissociation (k_5).

In the results shown in Figure 4 the molar serpin:trypsin ratio was 2:1 for wild-type α_1-PI and ($P_{12}P_7$OV)-α_1-PI; therefore, if some reactivation of trypsin was taking place it might not be measurable due to the excess serpin. Additional experiments were carried using a 1:2 ratio (α_1-PI:trypsin). In this case the maximum trypsin inhibition achieved was 50%; however, no regeneration of trypsin activity was detected indicating that the dissociation step, k_5, is very slow

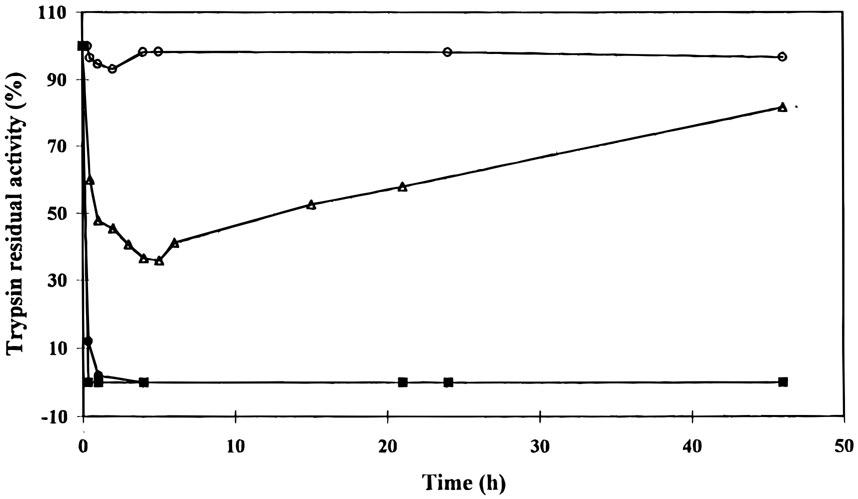

FIGURE 4: Stability of complexes between trypsin and α_1-PI determined by following residual trypsin activity after complexation, as a function of time. Reaction conditions are as described under Materials and Methods: (■) wild-type α_1-PI and trypsin, ratio 2:1; (●) ($P_{12}P_7$-OV)-α_1-PI and trypsin, ratio 2:1; (△) (P_6P_2OV)-α_1PI and trypsin, ratio 4:1; (○) ($P_{12}P_2$OV)-α_1-PI and trypsin, ratio 20:1.

for trypsin in complex with wild-type α_1-PI or ($P_{12}P_7$OV)-α_1-PI (results not shown). In these reactions trypsin was in excess and could potentially have degraded the complex causing the release of proteinase. There was no evidence that this occurred. Therefore it is likely that the regeneration of trypsin seen with (P_6P_2OV)-α_1-PI and ($P_{12}P_2$OV)-α_1-PI was due to dissociation of the complex into free enzyme and P_1—$P_{1'}$-cleaved serpin through a large increase in k_5. This was supported by the SDS gel (Figure 2) which shows that cleaved serpin is the product of these reactions. This is the first report indicating that residues in the reactive center loop, specifically those in the P_6—P_2 region, are important in determining k_5. This is an important finding as the crystal structure of a serpin—enzyme complex is not known, nor is it fully understood how deacylation is prevented. A possible reason for the lack of deacylation is that water is excluded from the proteinase active site in the complex and is therefore unavailable to act as a proton donor. The increased reactivation of the trypsin—(P_6P_2OV)-α_1-PI complex could be due to greater solvent accesibility than with wild-type α_1-PI. To address this issue, trypsin and (P_6P_2OV)-α_1-PI were incubated at pH 7.5 or 8.0 at the same concentration as in Figure 4, and at 4 h the incubations were supplemented with an equal volume of buffer, 2 M ammonium hydrochloride, or 2 M methylamine hydrochloride. If the acyl bond was solvent accessible the amine should be able to act as a nucleophile and increase the rate of deacylation of the complex. In no case was the rate of trypsin regeneration increased suggesting that solvent accessibility is not increased in the complex.

Overall the data show that the P_{12}—P_7 residues are important in recognition and initial reaction with the proteinase, presumably by affecting the conformation around the P_1—$P_{1'}$ bond. The P_{12}—P_7 residues also affect SI, but as the changes made in the variant were relatively conservative, they only resulted in a small increase in SI with HNE. The P_6—P_2 residues affect proteinase recognition and also have a very pronounced affect on SI and complex stability. Thus, either the P_6—P_2 residues alter the ability of the P_{12}—P_7 residues to insert or the rate of insertion of the P_6—P_2 residues themselves is much reduced. This would mean that insertion of the P_6—P_2 residues is critical for inhibition and complex stability. This is quite contrary to the view based on synthetic peptide insertion that only P_{14} insertion is critical for inhibition (Schulze et al., 1992) but consistent with data showing a considerable movement of the proteinase relative to the serpin in the stable complex (Stratikos and Gettins, 1997). Moreover, the situation is complicated by the finding that with complexes of (P_6P_2OV)-α_1-PI the rate constant k_5 is increased.

Kinetics of Cleavage of the Reactive Center Loop and Extent of Loop Insertion. A possible explanation for the increases in SI and k_5 observed for (P_6P_2OV)-α_1-PI and ($P_{12}P_2$OV)-α_1-PI is that cleavage had not occurred at the P_1—$P_{1'}$ bond but elsewhere in the reactive center loop or elsewhere on the protein. This hypothesis was tested by analysis of the new N-terminal sequences obtained when reacting (P_6P_2OV)-α_1-PI or ($P_{12}P_2$OV)-α_1-PI with HNE (Figure 6, lanes 5 and 2, respectively). With (P_6P_2OV)-α_1-PI, in addition to the amino terminus of the protein, the sequences DAMSIP and SIPPEV were found in a 7:3 molar abundance, respectively, indicating that cleavage occurred at both the P_4—P_3 and P_1—$P_{1'}$ bonds, with a greater efficiency of cleavage for the P_4—P_3 bond. The reaction mixture was run over a Mono-Q column, and the cleavage products were separated (Figure 5A). The native protein eluted at 23.2 min, product A at 25.6 min, and product B at 28.3 min. Amino-terminal sequencing showed that product A corresponded to P_4—P_3 cleaved (P_6P_2OV)-α_1-PI (Figure 6, lane 6) and that product B was P_1—$P_{1'}$ cleaved (P_6P_2OV)-α_1-PI (Figure 6, lane 7). Thus, for this variant part of reduced inhibitory activity is due to cleavage at a site other than P_1—$P_{1'}$.

Another possible reason for reduced inhibitory activity is incomplete loop insertion into β-sheet A. If partial loop insertion can account for the observed increase in SI for (P_6P_2OV)-α_1-PI, then the purified P_1—$P_{1'}$ cleaved product (product B) could be further cleaved at P_4—P_3 by HNE and a third product (double cleaved) would appear concomitant with a decrease in B. On the other hand, if full loop insertion had taken place, additional cleavage of B would not be possible and the formation of A and B would occur concurrently, reach a plateau, and follow the model for parallel reaction kinetics. The time course digestion of (P_6P_2-OV)-α_1-PI carried out under pseudo-first-order conditions showed disappearance of the uncleaved protein over time coincident with a parallel increase in products A and B

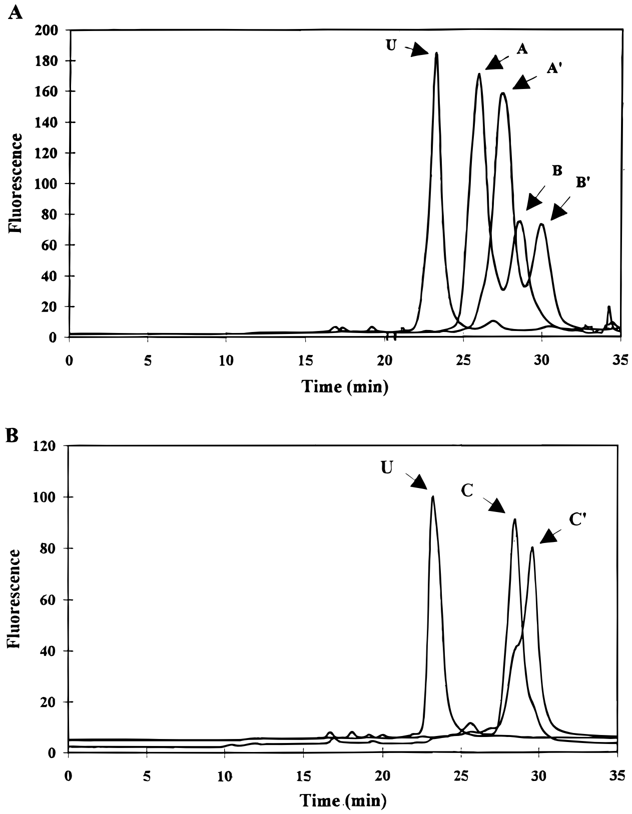

FIGURE 5: (A) Representative elution profiles of (P$_6$P$_2$OV)-α_1-PI and its cleavage products by HNE from a Mono-Q column. Each profile corresponds to 20 μg of the variant applied in 5 mM sodium phosphate, pH 7.0, and eluted with a linear gradient to 150 mM sodium phosphate, pH 7.0, after different times of digestion by HNE at 37 °C in 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000. Proteins were detected in-line by tryptophan fluorescence emission. U refers to uncleaved (P$_6$P$_2$OV)-α_1-PI; A and B refer to (P$_6$P$_2$OV)-α_1-PI cleaved at the P_4-P_3 and $P_1-P_{1'}$ bonds, respectively (after 45 min); A′ and B′ species correspond to A and B further cleaved in the amino-terminal extremity of the protein at position N-terminal + 7 (after 20 h). A′ has also been cleaved at the P_1-P_1' bond. (B) Elution profiles of (P$_{12}$P$_2$OV)-α_1-PI and its cleavage products by HNE from a Mono-Q column (same conditions as in panel *A*) after different times of digestion by HNE at 25 °C in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 10 mM CaCl$_2$, and 0.1% PEG 8000. U refers to uncleaved (P$_{12}$P$_2$OV)-α_1-PI, C to (P$_{12}$P$_2$OV)-α_1-PI cleaved at the $P_1-P_{1'}$ bond (after 30 min), and C′ to C further cleaved in the amino terminus at the same site previously described for A′ and B′ (after 1 h).

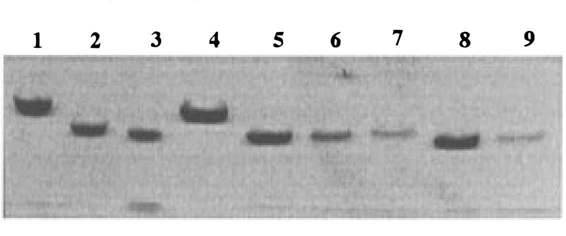

FIGURE 6: SDS gel (10%) of the purified cleavage products of (P$_6$P$_2$OV)-α_1-PI and (P$_{12}$P$_2$OV)-α_1-PI: lane 1, 2 μg of native (P$_{12}$P$_2$-OV)-α_1-PI; lanes 2 and 3, 2 μg of the cleaved (P$_{12}$P$_2$OV)-α_1-PI species C and C′; lane 4, native (P$_6$P$_2$OV)-α_1-PI; lane 5, 2 μg of 45 min digestion reaction mixture of (P$_6$P$_2$OV)-α_1-PI by HNE before Mono-Q purification; lanes 6 and 8, 2 μg of the cleaved (P$_6$P$_2$OV)-α_1-PI species A and A′; lanes 7 and 9, 1 μg of the cleaved (P$_6$P$_2$OV)-α_1-PI species B and B′.

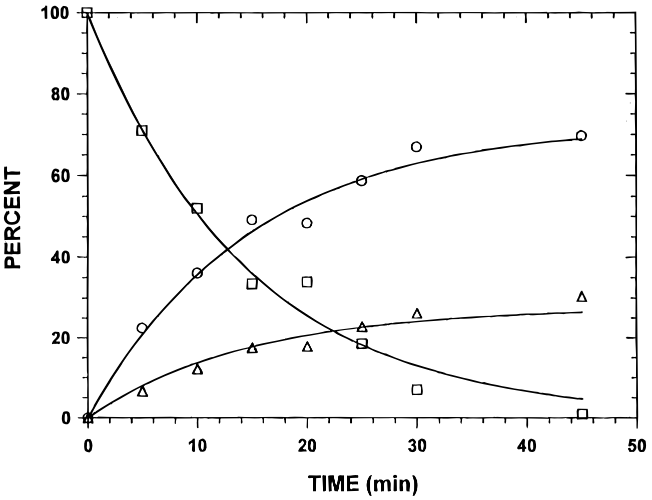

FIGURE 7: Cleavage of (P$_6$P$_2$OV)-α_1-PI by HNE: extent of loop insertion tested by the accessibility of the P_4-P_3 bond to HNE proteolysis. Conditions are as described under Materials and Methods. Normalized fluorescence intensities from peak area analysis of the Mono-Q-eluted species are represented as a percent of total species present. Native (P$_6$P$_2$OV)-α_1-PI (□), P_4-P_3 single-cleaved (P$_6$P$_2$OV)-α_1-PI (○), or $P_1-P_{1'}$ single-cleaved (P$_6$P$_2$OV)-α_1-PI (△) as deduced from peak areas of species A or B, respectively, in Figure 5A and data modeled according to parallel reaction kinetics.

(Figure 7). This parallel reaction mechanism behavior clearly indicates that once the $P_1-P_{1'}$ bond is cleaved in (P$_6$P$_2$OV)-α_1-PI there is no further cleavage within the reactive center loop and hence suggests complete loop insertion upon cleavage, at least up to P$_3$. Additional cleavage of A and B was tested by letting the reaction proceed for 20 h. Under these conditions A and B were digested further to give products A′ and B′ (Figure 5A; Figure 6, lanes 8 and 9), which were present in the same relative amounts as A and B. The N-terminal sequences present in peak A′ were DAMSIP, SIPPEV, and DTSHHD. These correspond to the sequences starting at P$_3$, P$_{1'}$, and residue 7 in the amino terminus, respectively. Thus, peak A (which was cleaved at P_4-P_3) was further cleaved in the reactive center at $P_1-P_{1'}$ because this bond would remain exposed at the “top” of the molecule attached to sheet 1C and could be cleaved with loss of the P_3-P_1 tripeptide. This material and the material starting at P$_3$ were also cleaved at an additional site within the amino terminus. Sequences present in peak B′ were SIPPEV and DTSHHD; therefore, additional cleavage occurred only in the amino-terminal region with no cleavage at P_4-P_3, indicating that this bond was no longer accessible. Taken together, these results strongly support complete loop insertion upon cleavage of the (P$_6$P$_2$OV)-α_1-PI variant by HNE.

From the data in Figure 7 the observed pseudo-first-order rate constant for the formation of A (cleavage at P_4-P_3) was calculated to be 0.049 min^{-1} and for B to be 0.019 min^{-1}. Since formation of A was 2.5-fold faster than formation of B (cleavage at $P_1-P_{1'}$), and B cannot further be cleaved at the P_4-P_3 bond presumably due to loop insertion, this suggests that loop insertion must occur at least 10 times faster than cleavage at the P_4-P_3 bond; otherwise we would have found some evidence for a decrease in B and conversion into a double-cleaved product. This gives a lower limit for the rate constant of loop insertion of 0.49 min^{-1} or 0.008 s^{-1}. This is 400 times lower than the rate of loop insertion during the formation of the t-PA-PAI-1 complex which is reported to be 3.4 s^{-1} (Shore et al., 1995). Even though loop insertion in B occurs fast enough to prevent further cleavage, it is probably too slow to “lock” the complex and

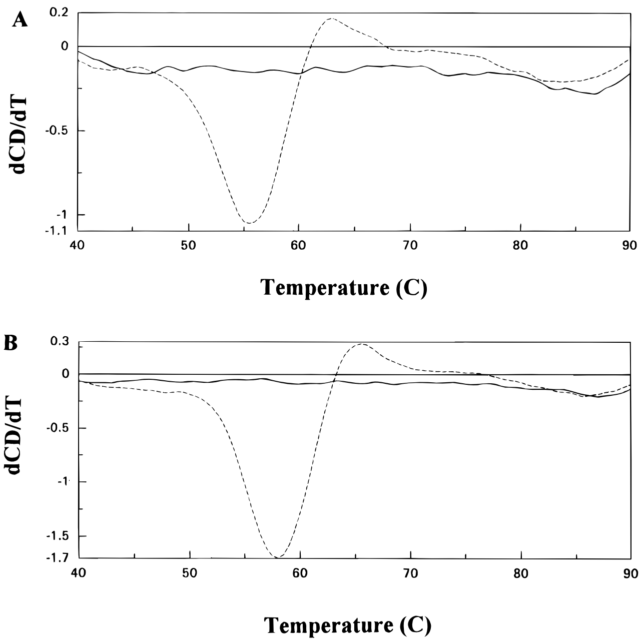

FIGURE 8: Thermal stability of native and cleaved α_1-PI determined by circular dichroism: (A) heat denaturation of native (dotted line) and papain-cleaved (solid line) wild-type α_1-PI and (B) heat denaturation of native (dotted line) and cleaved (solid line) (P$_{12}$P$_2$OV)-α_1-PI. The data are represented as the first derivative of mdeg as a function of temperature, dθ/dT. The denaturation temperature was taken at the minimum point of the curve.

prevent deacylation. Thus, with (P$_6$P$_2$OV)-α_1-PI there are four possible reasons for the reduced inhibitory activity: (i) reduced rate of initial interaction with proteinase due to alterations in K_s and/or k_2, (ii) preferential cleavage at the P$_4$–P$_3$ bond, (iii) reduced rate of loop insertion leading to an increase in SI, and (iv) reduced stability for any complex which does form due to an increase in k_5.

The HNE cleavage site in (P$_{12}$P$_2$OV)-α_1-PI was also determined. For this, six rounds of amino-terminal sequencing of the reaction mixture of the protein cleaved by HNE were carried out (Figure 5B, product C; Figure 6, lane 2). The sequences found were the N-terminus of the variant and the sequence SIPPEV. This corresponds to residues P$_{1'}$–P$_{6'}$ hence cleavage had only occurred at the P$_1$–P$_{1'}$ bond. Although both (P$_{12}$P$_2$OV)-α_1-PI and (P$_6$P$_2$OV)-α_1-PI have the same P$_4$–P$_3$ residues, no cleavage at this site in the former was found. Incubation with more HNE caused additional cleavage within the amino terminus (DTSHHD) which is the same additional site previously found in (P$_6$P$_2$OV)-α_1-PI, but no further cleavage within the reactive center was found (Figure 5B, product C′; Figure 6, lane 3). These data give further evidence that the residues in the P$_{12}$–P$_7$ region can affect the residues in the P$_6$–P$_2$ region, presumably by altering their conformation. The proteinase accessibility of bonds in the reactive center loop is therefore very sensitive to the local conformation. In support of this, cleavage within the reactive center loops of ovalbumin, antithrombin, and PAI-1 can be altered by localized conformational changes (Huntington et al., 1995, 1996; Kjøller et al., 1996).

Heat Stability of Native and Cleaved (P$_{12}$P$_2$OV)-α_1-PI. To show that (P$_{12}$P$_2$OV)-α_1-PI could undergo loop insertion on cleavage, heat denaturation was followed by loss of elipticity at 222 nm using circular dichroism spectroscopy. Figure 8A shows the expected behavior of wild-type α_1-PI, with the native protein denaturing at 55.5 °C and the reactive center loop-cleaved (papain-cleaved) material being stable up to 90 °C. The same increase in heat stability was seen when the (P$_{12}$P$_2$OV)-α_1-PI was cleaved, with the denaturation temperature going from 57.5 °C for the native to greater than 90 °C for the cleaved (Figure 8B). This is consistent with the reactive center loop inserting into β-sheet A. The 2 °C greater stability of the native variant compared to wild-type presumably reflects a difference in the conformation of the reactive center loop. In wild-type α_1-PI the sheet conformation of the reactive center loop is stabilized by a hydrogen bond from the P$_5$ Glu to Arg^{196} (Elliott et al., 1996). In (P$_{12}$P$_2$OV)-α_1-PI the P$_5$ residue is Gly, and this stabilization cannot take place. In ovalbumin the reactive center loop is helical and is stabilized by salt bridges from P$_7$ Glu and P$_3$ Asp to Lys^{283} (Stein et al., 1991). In α_1-PI the equivalent residue is not Lys but Ser; consequently, this interaction cannot take place in (P$_{12}$P$_2$OV)-α_1-PI. However, since the P$_5$ hydrogen bond cannot occur in this variant, the reactive center loop might still be in a helical conformation, as this might be the most stable conformation for this region to adopt in this particular context (Patston & Gettins, 1996).

CONCLUSIONS

A number of conclusions can be made from the data presented here. Firstly, the reactive center loop residues P$_{12}$–P$_2$ of ovalbumin can be present in inhibitory serpins. Although the rates of inhibition with (P$_{12}$P$_7$OV)-α_1-PI and (P$_6$P$_2$OV)-α_1-PI are reduced relative to wild-type-α_1-PI, they are no worse than those found with some physiological serpin–enzyme reactions such as C1 inhibitor with kallikrein and factor XIIa or protein C inhibitor with activated protein C. Therefore, these residues are probably only partially responsible for the inhibitory defect in ovalbumin, which agrees with the data of Huntington et al. (1997) and McCarthy and Worrall (1997). Secondly, the P$_{12}$–P$_7$ and P$_6$–P$_2$ residues affect not only the rate of loop insertion but also the initial interaction with proteinase, these effects being nonadditive. Presumably this occurs as a result of conformational changes transmitted throughout the reactive center loop.

The third, and major, finding from these studies is that the residues in the P$_6$–P$_2$ region affect k_5 and complex stability. It is not known how the serpin–proteinase complex is stabilized, although it is presumed that deacylation is prevented by distortion of the catalytic triad (Plotnick et al., 1996). This might be the result of conformational changes on both the serpin and the proteinase (Kaslik et al., 1997; Stavridi et al., 1996). The P$_2$ Pro to Ala variants of α_1-PI-Arg^{358} produced previously (Heeb et al., 1990; Patston et al., 1990; Hopkins et al., 1995) were good inhibitors of many proteinases suggesting that an Ala at P$_2$ would not be the reason for the behavior of the variants studied here. The extent of loop insertion in the complex is not known although fluorescence data on the α_1-PI–trypsin complex show that both partial and full insertion of the loop are feasible (Stratikos & Gettins, 1997). In the fully loop-inserted serpins the P$_6$ and P$_4$ residues are oriented toward the center of the molecule rather than the solvent (Loebermann et al., 1984). If this same arrangement is present in the complex then these residues cannot be interacting with the proteinase. This suggests that the P$_5$ and P$_3$ residues could be in contact with the proteinase in the complex and be participating in the stabilization. An alternative explanation is that the P_5 and P_3 residues are not in contact with the proteinase but are in contact with other serpin residues. Thus, in cleaved α_1-PI (Loeberman et al., 1984), P_5 Glu is in close proximity to Leu^{176} and Asp^{177} in the loop connecting helix F to sheet 3A and to Ser^{330} and Lys^{331} in sheet 5A. Similarly, P_3 Ile is in close proximity to Asp^{179} in the loop connecting helix F to sheet 3A and to Lys^{328} and Ser^{330} in sheet 5A. Interactions such as these might be important in stabilizing the cleaved form of the serpin in the complex.

In the absence of crystallographic or other structural data on serpin−proteinase complexes, the nature of the complex remains open to speculation. However, by correlating the available structural data on serpins and our results, a plausible mechanism for stabilization of serpin−proteinase complexes can be proposed. Stable serpin−proteinase complex formation requires conformational changes on both molecules. The P_6−P_2 residues are critical because insertion of the side chains of the P_6 and P_4 amino acids is important in stabilizing the serpin conformational change. The P_5 and P_3 residues might interact with the proteinase to assist in the distortion of the active site, or they might interact with adjacent serpin residues which helps to stabilize the loop-inserted form. These are important issues, and future studies in this area will enhance our understanding of serpin mechanism and complex stability.

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