Evidence for the Extent of Insertion of the Active Site Loop of Intact α_1 Proteinase Inhibitor in β-Sheet A

Andreas J. Schulze,* Paul W. Frohnert, Richard A. Engh, and Robert Huber

Max-Planck-Institut für Biochemie, W-8033 Martinsried bei München, Germany

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ABSTRACT: The extent of insertion of β-strand s4A into sheet A in intact serpin α_1-proteinase inhibitor (α_1PI) has been probed by peptide annealing experiments [Schulze et al. (1990) *Eur. J. Biochem. 194*, 51–56]. Twelve synthetic peptides of systematically varied length corresponding in sequence to the unprimed (N-terminal) side of the active site loop were complexed with α_1PI. The complexes were then characterized by circular dichroism spectroscopy and tested for inhibitory activity. Four peptides formed complexes which retained inhibitory activity, one of which was nearly as effective as the native protein. Comparison with the three dimensional structures of cleaved α_1PI [Löbermann et al. (1984) *J. Mol. Biol. 177*, 531–556] and plakalbumin [Wright et al. (1990) *J. Mol. Biol. 213*, 513–528] supports a model in which α_1PI requires the insertion of a single residue, Thr345, into sheet A for activity.

The serpin superfamily consists mainly of serine proteinase inhibitors which play important roles in blood coagulation, inflammation, and fibrinolysis. Its members, including the prototypic α_1PI,[1] are characterized by a high degree of sequence homology and by implication possess a common tertiary structure (Huber & Carrell, 1989). This has been corroborated by three-dimensional structures of several proteolytically modified serpins, α_1PI (Löbermann et al., 1984), α_1PI S-variant (Engh et al., 1989), α_1-antichymotrypsin (Baumann et al., 1991), plakalbumin (Wright et al., 1990), and the intact noninhibitory ovalbumin (Stein et al., 1990). We have previously demonstrated that incubation of α_1PI with a synthetic peptide identical in sequence to the unprimed site of the active site loop residues 345–358 generates a complex which mimics the cleaved molecule (Schulze et al., 1990). The binary complex is noninhibitory and acts instead as a proteinase substrate. The structure of plakalbumin, and subsequently ovalbumin (Stein et al., 1990), provided a model for an uncleaved serpin (Wright et al., 1990; Engh et al., 1990) with a 5-stranded β-pleated sheet A bridged to β-sheet C by the active site loop. After proteolytic modification of α_1PI by its target proteinase, P1 Met and P1′ Ser are separated by 70 Å as the unprimed active site loop is inserted as strand s4A in an antiparallel 6-stranded β-sheet A. Circular dichroism spectroscopy and fluorescence emission studies have been used to distinguish between the uncleaved and cleaved conformation of serpins (Bruch et al., 1988; Schulze et al., 1990). To date, no X-ray structure of an uncleaved inhibitory member of the serpin family has been determined.

All inhibitory serpins have a small residue (Thr, Ser, Ala, or Val) at position 345 in strand s4A which is buried in the cleaved species. Ovalbumin and its proteolytically modified form plakalbumin, noninhibitory serpins, have an arginine in position 345 which for stereochemical reasons cannot be internal. This was suggested as the main reason for the lack of conformational transition upon insertion of strand s4A into sheet A (Wright et al., 1990; Schulze et al., 1990). The recently characterized recombinant α_1PI-Thr345→Arg,Met358→Arg variant (Schulze et al., 1991) is not an inhibitor and is similar to ovalbumin concerning denaturation stability, demonstrating the key role of residue 345 for both inhibitory activity and the rearrangement of sheet A. We concluded that partial insertion of strand s4A is necessary for inhibitory conformation of the binding loop. A bulky residue at position 345 prevents this and renders the inhibitor a substrate. The loop adopts an α-helical conformation in intact ovalbumin (Stein et al., 1990), not competent for proteinase binding (Bode & Huber, 1991). It has been suggested also that active serpin inhibitors have an α-helical loop which uncoils prior to or during proteinase attachment accompanied by insertion of strand s4A into sheet A up to residue 349 (P10) (Skriver et al., 1991).

Several studies indicate a third conformation of serpins, called the latent (L-) form, which is not inhibitory. PAI-1 spontaneously adopts the L-form in vitro (Hekman et al., 1985), and Carrell et al. (1991) demonstrated that also antithrombin III seems to be able to adopt this particular conformation. The three-dimensional structure of the latent form of PAI-1 (Mottonen et al., 1992) provides a structural basis for the phenomenon of latency but cannot provide direct information about the structure of an active serpin inhibitor. It gives further evidence of the flexibility of the molecule, in particular in sheets A and C. No comparable latent state has been described for α_1PI.

Although there is significant evidence that the active inhibitory conformation includes partial insertion of strand s4A into sheet A, its extent is unclear. By integration of a series of peptides mimicking progressively shorter lengths of the unprimed active site loop into β-sheet A, it seemed promising to probe the extent of insertion by monitoring the onset of inhibition. C-terminally truncated peptides (beginning with Thr345) provide a control for the experiment.

In all, 9 N-terminally and 3 C-terminally truncated peptides (Table I) were synthesized and complexed with α_1PI, and their inhibitory activity and CD spectra were measured. The results indicate that insertion of only Thr345 is sufficient for inhibitory activity.

* Corresponding author.

[1] Abbreviations: α_1PI, α_1-proteinase inhibitor; α_1PI*, cleaved inhibitor; b.c., binary complex; CD, circular dichroism; FAB-MS, fast atom bombardment mass spectrometry; F-moc, 9-fluorenylmethyloxycarbonyl; PAGE, polyacrylamide gel electrophoresis; ppE, porcine pancreatic elastase.

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Table I: Summary of Synthesized Peptides

total no. of residues	sequence	code
	N-Terminally Truncated Peptides	
14	Ac-TEAAGAMFLEAIVM-OH	I
12	Ac-AAGAMFLEAIVM-OH	II
12	F-AAGAMFLEAIVM-OH	III
11	Ac-AGAMFLEAIVM-OH	IV
11	IBu-AGAMFLEAIVM-OH	V
10	Ac-GAMFLEAIVM-OH	VI
9	Ac-AMFLEAIVM-OH	VII
8	Ac-MFLEAIVM-OH	VIII
7	Ac-FLEAIVM-OH	IX
	C-Terminally Truncated Peptides	
11	Ac-TEAAGAMFLEA-OH	X
7	Ac-TEAAGAM-OH	XI
4	Ac-TEAA-OH	XII

MATERIALS AND METHODS

α_1PI was provided by Prof. C.-B. Laurell and was isolated from human plasma as previously described (Schulze et al., 1990).

Peptide Synthesis. Solid-phase peptide synthesis was carried out in a LKB Biolynx Synthesizer (Pharmacia LKB, Uppsala, Sweden) using the F-moc method (Atherton et al., 1987; Fields et al., 1990) starting with a F-mocMet resin. Peptides were N-terminally acetylated (Ac), formylated (F), or protected by an isobutyric acid group (IBu) (Table I). Purification followed on a Sephadex G 25 column in 0.1 M NH_3. Homogeneity was checked by FAB-MS and amino acid analysis.

Binary Complex Preparation. Peptide integration using a 200-fold molar excess of peptide over α_1PI and purification of the binary complexes were done as described (Schulze et al., 1990). α_1PI concentration was 2 mg/mL.

Circular Dichroism Spectroscopy. Circular dichroism spectroscopy was carried out in a Jobin Yvon Autodichrograph Mark IV (ISA Instruments S.A. Lonjumeau, France) using 5 mM sodium/potassium phosphate, pH 7, as buffer. Spectra were recorded from 205 to 250 nm and 250 to 340 nm, respectively, and processed as described in Schulze et al. (1990, 1991). Spectra are given in θ = deg cm^2 dmol^{-1}.

Polyacrylamide Gel Electrophoresis (PAGE). PAGEs to follow the peptide insertion were run on native 10% gels using the Pharmacia PHASTSystem (Pharmacia, Uppsala, Sweden).

Binary complexes were incubated with different proteinases in a molar ratio of 1:1 and separated on 14% SDS–PAGEs to check complex formation as a second indication for inhibitory activity. Proteinase and binary complexes were incubated at room temperature for 5 min (trypsin, porcine pancreatic elastase) and 7 min (thrombin). The proteolytic reaction was stopped by mixing with Laemmli buffer (Laemmli, 1970) and heating for 3 min at 95 °C.

Antitryptic Activity. Antitryptic activity was measured as residual trypsin activity employing 25 mM *N*-α-benzoyl-L-arginine-4-nitroanilide (Boehringer Mannheim, Germany) in dimethyl sulfoxide as the chromogenic substrate in a Beckman diode array spectrometer DU7400 (Beckman Instruments, Fullerton, CA). The detection wavelength was 405 nm. Activity measurements were done in 5 mM sodium/potassium phosphate, pH 7, after 5 min of preincubation of trypsin and binary complex in a molar ratio of 1:1 at room temperature. Results are plotted as the percentage of residual trypsin activity.

RESULTS

Complex Formation

Peptides and human α_1PI were incubated at 37 °C with 10 mM DTT to prevent oxidation of Met358 in 20 mM Tris-HCl, pH 8. Completeness of complex formation was checked on native 10% PAGE (Figure 2) by observing the shift of the complex band toward the anode due to the additional negative charges of the peptide [for further details see Schulze et al. (1990)]. Incubation was continued until 100% completion. Excess peptide was removed by gel filtration. A stoichiometry of each binary α_1PI–peptide complex was judged as 1:1 by amino acid analysis. The complexation with the peptides V, IX, and XII (not shown on the native gel in Figure 2) did

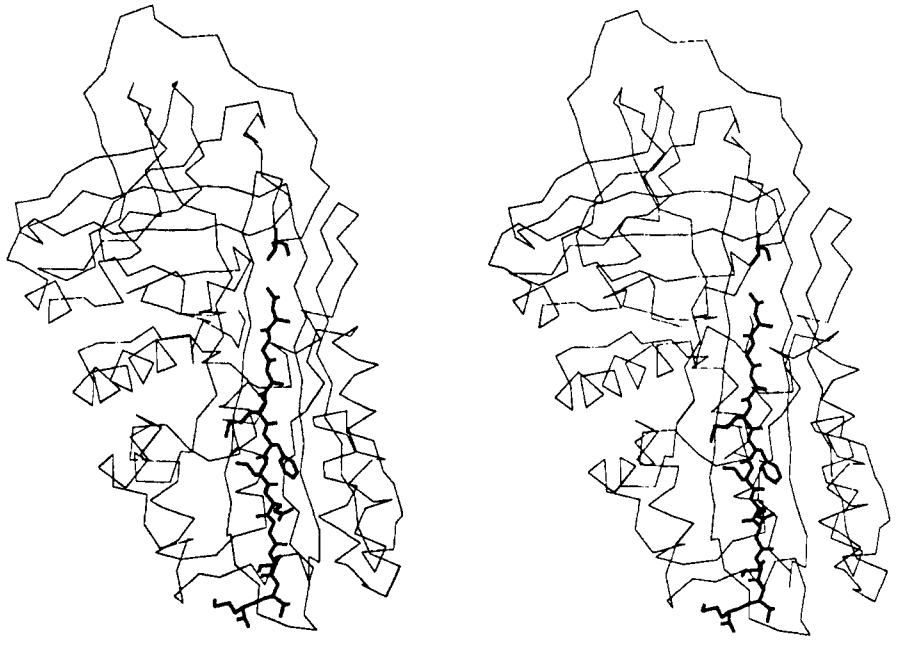

FIGURE 1: Stereo drawing of a model of the binary complex IV with the binding loop in "canonical conformation". β-sheet A is expanded by peptide IV. The C_α-chain of α_1PI is drawn in thin lines, Thr345 and peptide IV are drawn in thick lines with all atoms.

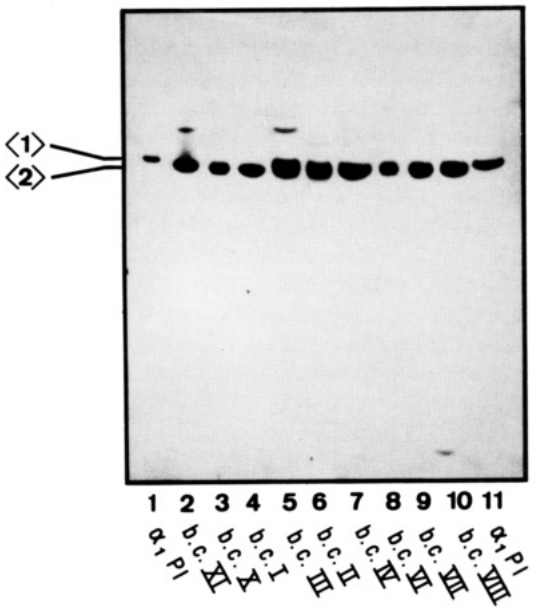

FIGURE 2: Native, 10% PAGE of the binary complexes compared to the native α_1PI. Band ⟨1⟩ is native α_1PI; band ⟨2⟩I represents binary complexes. Lanes 1, and 11, native α_1PI; lane 2, bc XI; lane 3, bc X; lane 4, bc I; lane 5, bc III; lane 6, bc II; lane 7, bc IV; lane 8, bc VI; lane 9, bc VII; lane 10, bc VIII.

not go to completion. No homogeneous complex could be prepared because uncomplexed α_1PI and binary complexes could not readily be separated.

Circular Dichroism (CD)

In general, the CD spectra of the complexes of C-terminally truncated peptides differ from those formed with N-terminally truncated peptides, but spectra are similar within these groups.

C-Terminally Truncated Peptides. The near-UV spectra of the binary complexes of peptides X and XI are similar to those of the binary complex I described earlier (Schulze et al., 1990). The spectra also resemble the proteolytically modified α_1PI (α_1PI*) with its characteristic differences at 280 and 295 nm compared to that of native α_1PI (Figure 3B).

The far-UV CD spectra of complexes I, X, and XI are also similar (Figure 3A). The intensity of the native protein at the 220-nm band, marked by [−/−], differs from those of binary complexes I and XI or that of the cleaved inhibitor ([−//−]) by about 20% or by 11% for binary complex X in negative ellipticity.

The spectra suggest similar structures of the binary complexes I, X, XI, and α_1PI*.

N-Terminally Truncated Peptides. Figure 3D shows the near-UV CD spectra of the binary complexes formed with peptides II, III, IV, VI, VII, and VIII. It is apparent that binary complexes with peptides II, III, VI, and VII show properties of native α_1PI spectra at 260 and 265 nm. The intensities of the 280- and 295-nm bands, on the other hand, range between the native and cleaved form. The complexes with peptides IV and VIII exhibit very clearly the native, uncleaved form characteristics over a wide spectral range. Similar conclusions can be drawn from the far-UV spectra of these complexes (Figure 3C). The binary complexes II, III, VI, and VII show a significant increase in α and/or β structure, whereas binary complexes IV and VIII resemble most closely the native uncleaved form.

SDS–PAGE

Serpins and their target proteinases generally form long-lived SDS-stable complexes, while substrates do not. Figure 4 shows SDS–PAGEs of the different binary complexes incubated with trypsin (A, top left), human thrombin (B, top right), and porcine pancreatic elastase (C, bottom). In general, all binary complexes are most rapidly cleaved by porcine pancreatic elastase, followed by trypsin and then thrombin.

Binary complexes with C-terminally truncated peptides are substrates and therefore do not form the stable enzyme complex (Figure 4, band ⟨1⟩).

Binary complex I also has substrate properties. Binary complexes II and III form two cleaved-form bands (α_1PI*) when cleaved by thrombin, indicating a second thrombin cleavage site which becomes accessible after peptide insertion. Binary complex III forms two α_1PI* bands when cleaved by trypsin as well. Binary complex IV is the only species that forms SDS-stable complexes with each of trypsin, thrombin, and porcine pancreatic elastase. Binary complex VI forms a SDS-stable complex with thrombin and ppE, whereas binary complex VIII does only with ppE. All bands of SDS-stable binary complexes are weaker than the corresponding bands of α_1PI and the different proteinases. All binary complexes that are not represented in Figure 4A behaved like bc I, i.e., were substrates and are therefore not shown.

Inhibitory Activity

No binary complex with a C-terminally truncated peptide showed antitryptic activity (Figure 5). The same is true for the complex with the full-length peptide I and the N-terminally truncated peptides II and III. However, peptide complex IV exhibits >90% residual inhibitory activity. To a lesser extent, peptide complexes VI, VII, and VIII possess antitryptic activity ranging between 10% (binary complex VIII) and 31% (binary complex VI) (Figure 5). As a control, we incubated α_1PI without peptide and observed no significant change in inhibitory activity during 7 days of incubation at 37 °C. A summary of the data from the CD, complex formation, and inhibitory studies is given in Table II.

DISCUSSION

Interference with α_1PI may disrupt diverse physiological pathways and lead to pathological conditions including lung emphysema, septic shock, and possibly also mucoviscidose or cystic fibrosis as seen in carriers of abnormal mutant α_1PI with altered proteinase specificity (e.g., α_1PI-Pittsburgh; Owen et al., 1983) or plasma levels (e.g., S-variant; Engh et al., 1989). The precise mechanism by which α_1PI inhibits proteinases is uncertain as a consequence of the lack of structural information about intact inhibitory serpins and their complexes formed with proteinases. The only intact serpins which have produced analyzable crystals are noninhibitory forms whose s4A β-strands are stabilized either completely out of sheet A (ovalbumin; Stein et al., 1990) or completely inserted into sheet A (latent form; Mottonen et al., 1992). It is assumed that intact inhibitory serpins adopt an intermediate state of insertion, supposedly necessary to produce the "canonical conformation" of the binding loop obligatory for inhibition. The experiments described here address this question.

For two reasons, we assume that peptides bind to the position of strand s4A in the cleaved species: (1) binary complex I behaves similar to α_1PI* in terms of circular dichroism spectroscopy, denaturation stability, and tryptophan fluorescence emission; (2) peptide insertion is sequence specific (Schulze et al., 1990).

Three peptides, the short peptides IX and XII as well as the isobutyric acid (IBu) protected peptide V, did not insert properly to yield binary complexes useful for further inves-

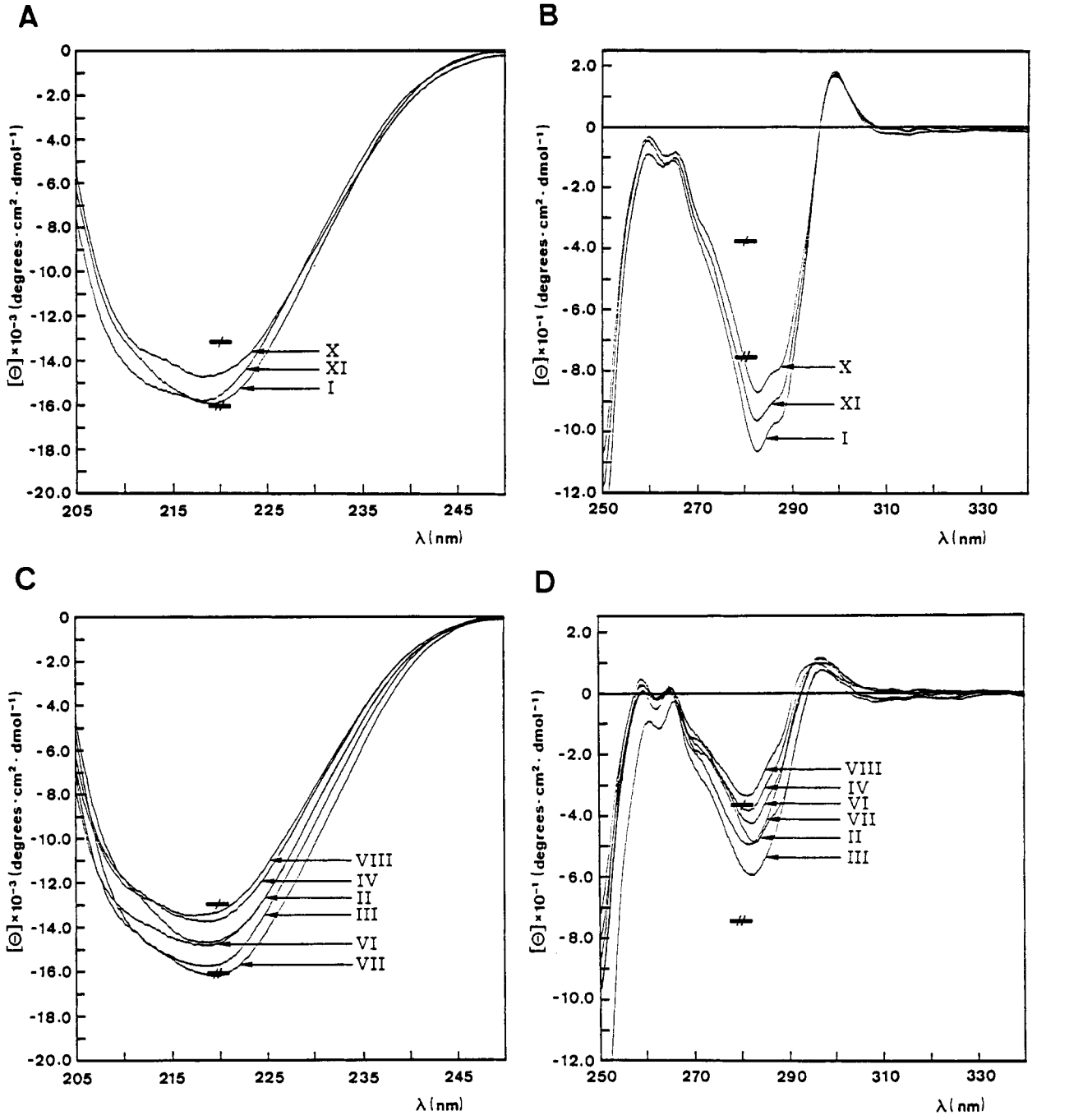

FIGURE 3: Far- and near-UV CD spectra of the binary complexes recorded from 205 to 250 nm (A, C) and 250 to 340 nm (B, D). Each of the binary complexes is marked by its numeric code as introduced in Table I. To facilitate understanding, intensities of native (-/-) and cleaved (-//-) α_1PI from Schulze et al. (1990) were marked at 220 nm and 280 nm.

tigation. Peptide V was not soluble under the incorporation condition. The peptides IX and XII are probably too short to insert stably. In addition, both lack the residue corresponding to Met351 (P8) in α_1PI. This residue is buried in a hydrophobic region between sheet A and the underlying α-helix B and likely plays an important role for the insertion reaction of the exogenous peptide and the stability of the complexes.

Each of the C-terminally truncated peptides begins with threonine (Thr345, P14 in α_1PI). The corresponding binary complexes exhibited no detectable antitryptic activity but are substrates (Figure 5). We assume that by insertion of these peptides the binding loop is completely exposed giving rise to a more stable noninhibitory, and possibly helical, conformation of this segment. On SDS–PAGE, no acyl-complex band can be detected and the α_1PI band is shifted to the lower molecular weight α_1PI* band position (Figure 4). Binary complexes X and XI show only small differences from I in near-UV CD spectra (Figure 3B), indicating similar environments at the aromatic chromophores (Trp194, Tyr244) near the active site loop.

The integration of the C-terminally truncated peptides apparently induces an overall tertiary structure similar to the cleaved form. Peptides I, X, and XI cause an increase in negative ellipticity of about 15% (220-nm band) comparable to the previously described increase from native to cleaved conformation (Bruch et al., 1988; Schulze et al., 1990).

In contrast, some N-terminally truncated peptides are apparently short enough to form complex structures which do not interfere with a partially inserted binding loop. Complex IV exhibits all the characteristics for such a complex, including an almost unchanged CD spectrum in the far- and near-UV

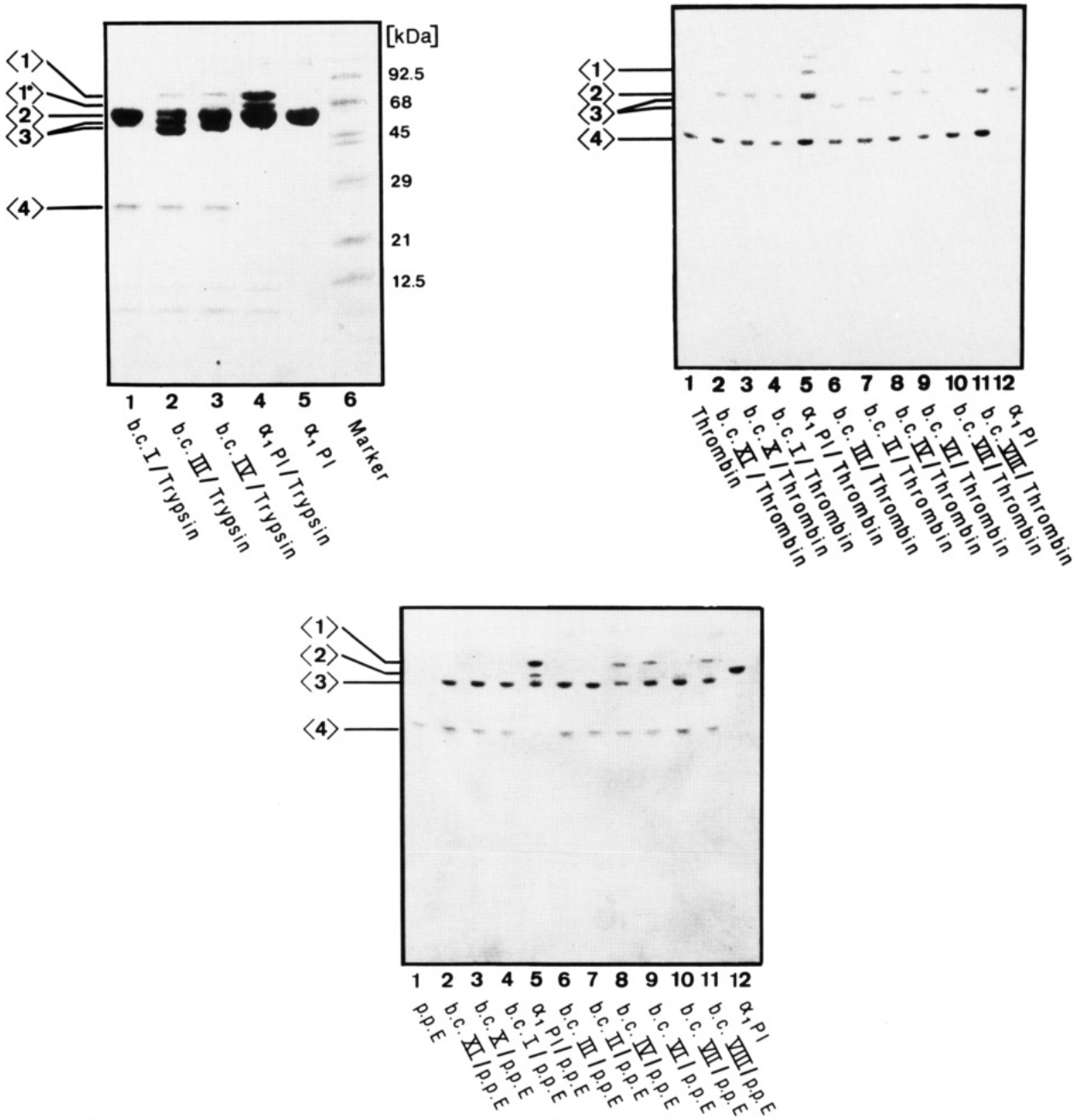

FIGURE 4: SDS–PAGEs of the binary complexes incubated with trypsin (A, top left), human thrombin (B, top right), and porcine pancreatic elastase (C, bottom). In all cases, an asterisk (*) means the proteolytically modified form. (A) Band ⟨1⟩, β-trypsin/α_1PI complex or β-trypsin/bc complex; band ⟨1*⟩, α-trypsin/α_1PI complex; band ⟨2⟩, native α_1PI or bc; band ⟨3⟩, α_1PI* or bc*; band ⟨4⟩, trypsin. Lane 1, bc I incubated with trypsin; lane 2, bc II incubated with trypsin; lane 3, bc IV incubated with trypsin; lane 4, α_1PI incubated with trypsin; lane 5, α_1PI; lane 6, molecular weight marker proteins as denoted. (B) Band ⟨1⟩, thrombin/α_1PI complex or thrombin/bc complex; band ⟨2⟩, α_1PI or bc; band ⟨3⟩, α_1PI* or bc*; band ⟨4⟩, thrombin. Lane 1, thrombin; lane 2, bc XI/thrombin; lane 3, bc X/thrombin; lane 4, bc I/thrombin; lane 5, α_1PI/thrombin; lane 6, bc III/thrombin; lane 7, bc II/thrombin; lane 8, bc IV/thrombin; lane 9, bc VI/thrombin; lane 10, bc VII/thrombin; lane 11, bc VIII/thrombin; lane 12, α_1PI. (C) Band ⟨1⟩, ppE/α_1PI complex or ppE/bc complex; band ⟨2⟩, α_1PI or bc; band ⟨3⟩, α_1PI* or bc*; band ⟨4⟩, ppE. Lane 1, ppE; lane 2, bc XI/ppE; lane 3, bc X/ppE; lane 4, bc I/ppE; lane 5, α_1PI/ppE; lane 6, bc III/ppE; lane 7, bc II/ppE; lane 8, bc IV/ppE; lane 9, bc VI/ppE; lane 10, bc VII/ppE; lane 11, bc VIII/ppE; lane 12, α_1PI.

(Figure 3C,D) which strongly indicates retention of the native inhibitory conformation. Only a slight increase in negative ellipticity at 220 nm (5%) was observed. Binary complex IV, in particular, shows near 100% inhibitory activity in the assay. Further evidence gave the SDS–PAGE band corresponding to the inhibitor/proteinase complex (76–81 kDa, depending on the proteinase). The band corresponding to the SDS-stable complex is weaker compared to that of the native α_1-PI/proteinase complex. A possible explanation might be that bc/proteinase complexes dissociate more rapidly when treated with SDS than the native α_1PI/proteinase complex. Dissociation induced by SDS treatment is common, and also α_1-PI/proteinase complexes, incubated in molar ratios with no residual tryptic activity, dissociate by SDS treatment.

Complexes II and III, however, are substrates (Figure 4) like the C-terminally truncated peptide complexes, presumably because their protecting groups interfere sterically with Thr345 and block partial insertion of the loop. Their CD spectra differ from cleaved and native α_1PI, indicating a change of environment around aromatic chromophores after peptide insertion. The aromatic residues near the binding loop and the insertion site are most likely to be affected.

The shorter peptides VI, VII, and VIII show shifts toward more negative ellipticities around the 220-nm band in the

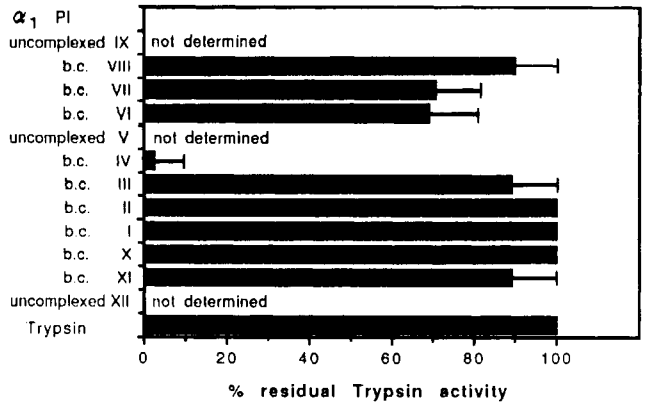

FIGURE 5: Inhibitory activity of native α_1PI and the binary complexes is expressed as residual trypsin activity in percentage compared to uninhibited trypsin set at 100%. Lane 1, trypsin, set at 100%; lane 2, bc XII; lane 3, bc XI; lane 4, bc X; lane 5, bc I; lane 6, bc II; lane 7, bc III; lane 8, bc IV; lane 9, bc V; lane 10, bc VI; lane 11, bc VII; lane 12, bc VIII; lane 13, bc IX; lane 14, α_1PI incubated without peptide.

Table II: Summary of All Results Obtained from CD Spectroscopy, Complex Formation with Proteinases, and Inhibition Studies Listed for All Binary Complexes

binary complex (no. of residues)	near-UV CD similarity to	far-UV CD similarity to	complex formation with			residual trypsin activity (100% − % of inhibitory activity)
			trypsin	thrombin	ppE	
With N-Terminally Truncated Peptides						
I (14)	bc	*/bc	–	–	–	100
II (12)	nat/*	*/bc	–	–	–	100
III (12)	nat/*	*/bc	×	–	–	~100
IV (11)	nat	nat	×	×	×	2.5
V (11)	–	–	–	–	–	nd
VI (10)	nat/*	*/bc	–	×	×	69
VII (9)	nat/*	nat/*	–	–	–	71
VIII (8)	nat	nat	–	–	×	90
IX (7)	–	–	–	–	–	nd
With C-Terminally Truncated Peptides						
X (11)	bc	*/bc	–	–	–	100
XI (7)	bc	*/bc	–	–	–	~100
XII (4)	–	–	–	–	–	nd

[a] Abbreviations and symbols: nd, not determined; nat, native inhibitor; bc, binary complex from Schulze et al. (1990); *, cleaved species; ×, yes; –, no.

far-UV CD spectra (Figure 3C). Their intensities range between native and cleaved α_1PI, and their inhibitory activity is reduced. This may be a result of distortion of the binding site loop possibly due to further insertion into sheet A promoted by gap formation between Thr345 and the peptide protecting group. We conclude from these data that the inhibitory form of α_1PI entails an insertion of a single residue, Thr345, into β-sheet A (Figure 1). All complexing peptides which have a Thr in position P14 block this insertion, rendering α_1PI a substrate. N-Terminally truncated peptides, long enough to interfere with the insertion of Thr345, behave similarly. Peptide IV, short enough to allow insertion of Thr345, creates a binary complex with the characteristics of the native inhibitor. Shorter N-terminally truncated peptides such as VI and VII also create inhibitory complexes, which are, however, with reduced activity and spectral characteristics intermediate between native and cleaved α_1PI forms.

Our results, interpreted with the structures of cleaved α_1PI and plakalbumin, are consistent with the model depicted in Figure 1.

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