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Modeling of Serpin-Protease Complexes: Antithrombin-Thrombin, α_1-Antitrypsin (358Met→Arg)-Thrombin, α_1-Antitrypsin (358Met→Arg)-Trypsin, and Antitrypsin-Elastase

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ABSTRACT **Based on the most recent available crystal structures and biochemical studies of protease complexes of normal and mutant serine protease inhibitors (serpins), we have built models of the complexes: α_1-antitrypsin + human neutrophil elastase; α_1-antitrypsin Pittsburgh (358Met→Arg) (Scott et al., J. Clin. Invest. 77:631–634, 1986) + trypsin; α_1-antitrypsin Pittsburgh (358Met→Arg) + thrombin; and antithrombin + thrombin. All serpin sequences correspond to human molecules.**

The models show correct stereochemistry and no steric clashes between protease and inhibitor. The main structural differences in the serpins from the parent structures are: (1) the reactive center loop is inserted into the A-sheet as far as P12; (2) strand s1C is removed from the C-sheet; and (3) the C-terminus has changed conformation and interacts with the protease.

In the absence of an X-ray structure determination of a serpin-protease complex, the demonstration that insertion of the reactive center loop into the A-sheet as far as P12 is stereochemically feasible provides structures of a protease-bound conformation of intact serpins with which to rationalize the properties of mutants, guide the design of experiments, and form a basis for further modeling studies, such as the investigation of the interaction of heparin with serpin-protease complexes.

Key words: serine-protease inhibitor, protease complex, modeling

INTRODUCTION

The serpin family of serine protease inhibitors is widely distributed in eukaryotes and includes the protease inhibitors responsible for the control of coagulation and other proteolytic cascades in the human.[1–3] The serpins are large monomeric proteins, typically containing 400–450 residues with a well-conserved tertiary structure formed by nine α-helices (A–I) and three β-sheets (A–C). A characteristic feature of the serpins is their ability to undergo major conformational changes. These involve rearrangements of a 27-residue region containing the active site residues. The first indication of the scale of these conformational rearrangements appeared in the crystal structure of cleaved α_1-antitrypsin, in which the ends of the cleaved bond are 65 Å apart[4] with the residues N-terminal to the cleavage site inserted as a strand into the large central β-sheet. Total incorporation of this region into the A-sheet also occurs in the latent conformation, as seen in the crystal structures of plasminogen activator inhibitor-1 (PAI-1)[5] and antithrombin.[6] Partial insertion is seen in an active form known from the crystal structure of antithrombin.[6,7] Table I summarizes the conformations seen in crystal structures.

A detailed understanding of the protease-serpin interaction requires the structure of a complex, but so far no crystal structure of a complex has been determined. However, the known structures of serpins and proteases, together with experimental studies of their interactions, make it possible to build models of complexes. Such models elucidate likely conformational constraints on serpins in their complexes with proteases, and details of the interactions between the protease and inhibitor. The models provide a basis for interpretation of the physiological consequences of mutations and support attempts to design drugs to counteract them.

In this section, we describe the experimental investigations bearing on the conformation of the pro-

Abbreviations: 1 Å = 10^{-10}m; FPA, fibrinopeptide A; 1kcal = 4.184 kJ; PDB, Protein Data Bank; PAI-1, plasminogen activator inhibitor-1; PPACK, D-Phe-Pro-Arg-chloromethylketone; r.m.s., root-mean-square; Serpin, serine-protease inhibitor (α_1-antitrypsin type). Residues in antithrombin are designated by their residue numbers in the antithrombin sequence, with the number of the equivalent α_1-antitrypsin residue shown in parentheses. α-Helices and β-sheets are denoted A, B, C, . . . in order of appearance in the polypeptide chain; strands of β-sheet are denoted snX where n = 1, 2, . . . and X = A, B, C.

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TABLE I. Crystal Structures of Serpins and Serine Proteinases

Name	Species	PDB code	No. of residues	Resolution Å	R-factor	State	Reference
Ovalbumin	Chicken	1ova	385	1.95	0.17	Native	8
Antichymotrypsin mutant	Human		400	2.5	0.21	Native	12
Antithrombin	Human	1ath	432	3.2	0.18	Native	6,7
PAI-1	Human		378	2.6	0.19	Latent	5
Antithrombin	Human	1ath	432	3.2	0.18	Latent	6
α_1-antitrypsin	Human	7api	393	3.0	0.19	Cleaved	4
Antithrombin	Cow	1att	433	3.2	0.21	Cleaved	43
Plakalbumin	Chicken		385	2.8	0.19	Cleaved	44
Antichymotrypsin	Human	2ach	400	2.7	0.18	Cleaved	45
Equine leucocyte elastase inhibitor	Horse	1hle	381	1.95	0.18	Cleaved	46
Human neutrophil elastase	Human	1hne	218	1.84	0.16	Complex	47
Trypsin	Cow	1tgs	229	1.8	0.18	Complex	48
Trypsin	Cow	2ptc	229	1.9	0.19	Complex	49

tease-bound form of intact serpins from which we have derived a set of features to build into our models. We then present the methods and results of model-building of the following four complexes: α_1-antitrypsin Pittsburgh (358Met→Arg)-thrombin, α_1-antitrypsin Pittsburgh (358Met→Arg)-trypsin, α_1-antitrypsin-elastase and antithrombin-thrombin. We used the mutant α_1-antitrypsin for the first two complexes because it, but not native α_1-antitrypsin, inhibits trypsin and thrombin.

General Description of the Serpin Structure and Its Conformational States

In this section, we illustrate the known serpin conformations and introduce nomenclature for the elements of the structure and the conformational changes.

Four structures illustrate the variations that can occur in the conformation of the reactive loop and the A-sheet (Fig. 1a–d).

Figure 1a shows the structure of cleaved α_1-antitrypsin.[4] Like other known serpin structures, it contains three sheets. The largest, the A sheet, is red, the B sheet green, and the C sheet yellow. Strand four of the A-sheet and strand one of the C sheet—the strands involved in the conformational changes—are purple. The two ends resulting from cleavage are marked by pink spheres. Figure 1b,c show the structures of ovalbumin[8] and native antithrombin.[6,7] In these structures, the A-sheet contains five strands. In both cases, the region around the active site, known as the reactive center loop (in purple) is at the top of the molecule. In ovalbumin, this region is completely out of the A-sheet and forms an α-helix. In one of the antithrombin molecules in the crystal structure, it is partially inserted into the A-sheet. It is this region that upon cleavage inserts into the A-sheet to form strand four (purple in Fig. 1a) of a six-stranded A-sheet. Figure 1d shows the structure of latent antithrombin—a six-stranded, uncleaved, inactive conformation.[6] Strand four of the A-sheet and the latent loop are in purple. The latent conformation is also seen in the crystal structure of plasminogen activator inhibitor-1 (PAI-1).[5]

The incorporation of the additional strand into the A-sheet requires conformational changes involving most of the molecule, known as the S→R transition. There is a relative movement of two fragments: a large fragment consisting of helices A, B, C, G, H, and I, sheets B and C, and strands s5A and s6A; and a smaller fragment consisting of helix F and strands s1A, s2A, and s3A.[9] Helices D and E and the top of strand s3A serve as flexible joints.

The Reactive Center Loop

Crucial to the function of serpins is the reactive center loop, comprising residues P16–P'14 in antithrombin.* This region contains the scissile bond (the peptide bond between 358 Met(P1) and 359 Ser(P'1) in α_1-antitrypsin); and the residues that fit into the cleft on the protease.

The reactive center loop is also involved prominently in the conformational changes of serpins. It includes the hinge region residues: P7 to P15,[11] which contain a segment of fairly well conserved sequence N-terminal to the scissile bond; in contrast P1 to P6 vary considerably in sequence among the inhibitory serpins. The reactive center loop also contains a consensus sequence at its amino terminus. In inhibitory serpins, residues P9 to P12 are usually small: (Ala, Gly, or Ser), P13 is usually Glu, P14

*In the notation of Schechter and Berger[10] residues are numbered starting at the scissile bond, i.e., the bond that the protease is poised to cleave. Residues N-terminal to the scissile bond are denoted P1, P2 . . . and those C-terminal to P1 are denoted P'1, P'2 . . . . The scissile bond itself is the peptide bond between residues P1 and P'1.

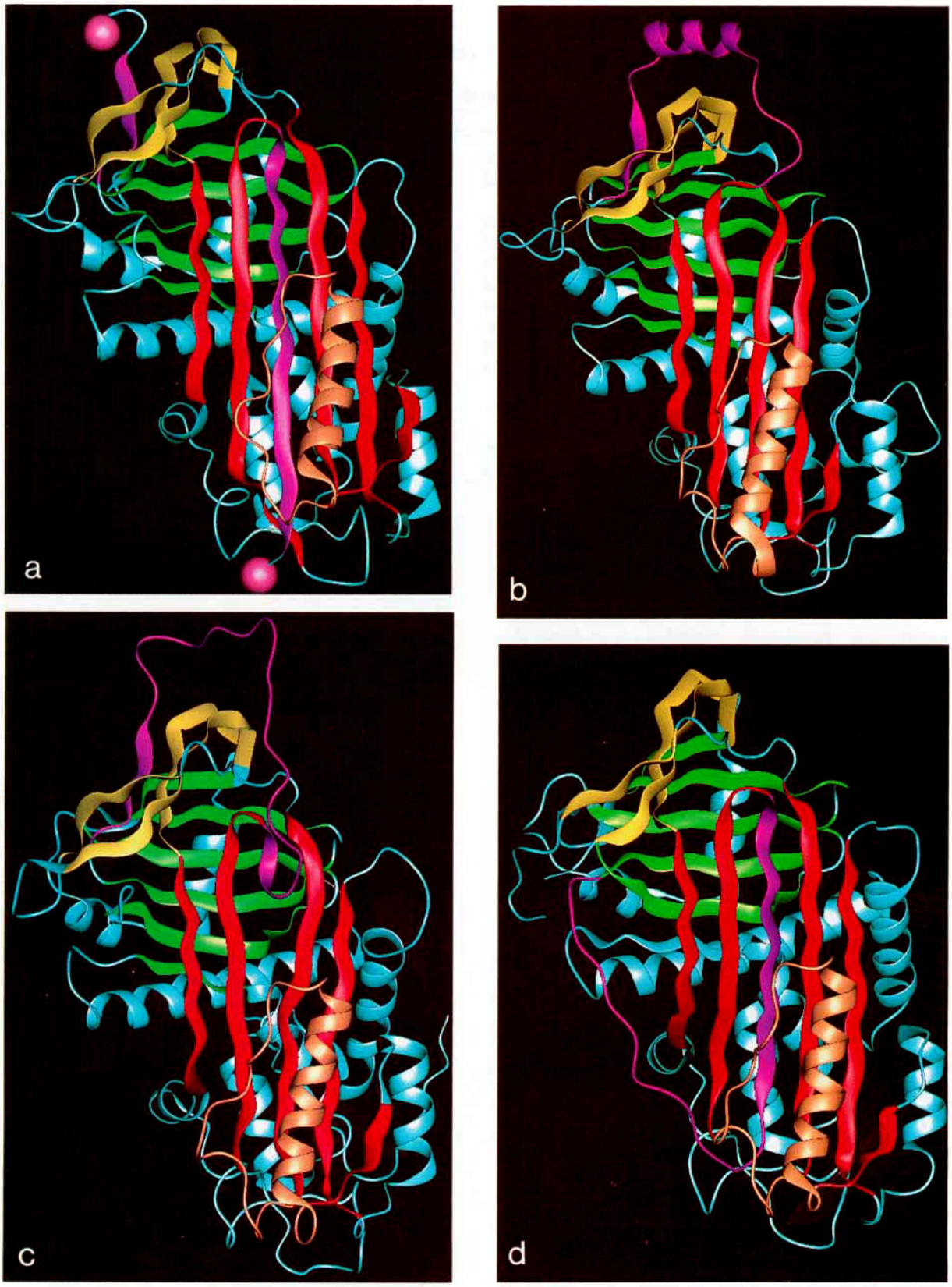

Fig. 1. Serpin conformations seen in crystal structures. **a:** Cleaved α_1-antitrypsin.[4] The A-sheet is colored red, the B-sheet green, and the C-sheet yellow. The F-helix and adjoining loops are colored brown. Strand four of the A-sheet and strand one of the C-sheet—the strands involved in the conformational changes—are colored purple. The two ends resulting from cleavage are shown in pink spheres. **b:** Ovalbumin.[8] In this structure, the A-sheet contains 5 strands. The β-sheets and F-helix are colored as in Figure 1a. The reactive center loop and s1C strand (in purple) can be seen at the top of the molecule. **c:** Native antithrombin.[6,7] The β-sheets and F-helix are colored as in Figure 1a. The reactive center loop and strand s1C (in purple) can be seen at the top of the molecule. **d:** Latent antithrombin—a six-stranded, uncleaved, inactive conformation.[6] The β-sheets and F-helix are colored as in Figure 1a. Strand four of the A-sheet and the latent loop are shown in purple.

TABLE II. Serpin Mutants and Their Functional Consequences

Serpin	Mutant	Effect	Reference
α_1-antitrypsin	P7 Glu	No effect	17
α_1-antitrypsin	P8 Glu	No effect	17
α_1-antitrypsin	P9 Glu	No effect	17
α_1-antitrypsin*	P10 Pro	15% inhibitory activity vs. trypsin, 4% inhibitory activity vs. elastase	10
α_1-antitryspin	P10 Glu	Slight destabilization of complex	17
C1 inhibitor†	P12 Glu (Ma)	Substrate of plasma Kallikrein	16
α_1-antitrypsin	P12 Glu	Predominantly substrate	17
Antithrombin	P12 Thr (Hamilton)	Predominantly substrate	24
α_1-antitrypsin	P14 Arg	Predominantly substrate	50
C1 inhibitor	P14 Glu (We)	Substrate	51

*The P10 proline mutant underwent the S→R transitions. Note that it is unlikely that the proline can insert into the non-edge strand of the β-sheet, because the normal hydrogen bonds cannot be formed.
†Antibodies that recognize cleaved and complexed (wild- type) C1 inhibitor do not recognize cleaved C1 (Ma) inhibitor, indicating that the S→R transition was not fully complete.

small (Ser, Thr, or Val) and P15 is usually Gly. Several point mutants in this region show loss of inhibitory activity (see below). This region can be considered as the proximal hinge of the conformational change of the loop. The C-terminus of the reactive center loop joins the s1C strand which links it to the B-sheet and forms the distal hinge.

Three conformations of the reactive center loop are seen in crystal structures of inhibitory serpins. In one molecule of antithrombin, residues P15–P14 are inserted into the A sheet. In the structure of a mutated antichymotrypsin, no residues are inserted.[12] (The inference that strand s1C acts as a distal hinge of the loop is based on crystal structures showing significant movement of this strand.) In the latent forms of PAI-1 and antithrombin, strand s1C is entirely displaced in order to allow full insertion of the reactive center loop into the A-sheet. In the cleaved state of antithrombin and α_1-antitrypsin residues P15–P3 are fully inserted into the A-sheet, as in the latent form.

Considerations for Design of Models

The conformation of intact serpins, in complex with a protease, is expected to be similar but not identical to the related conformation seen in crystal structures. It should be noted that the structures observed in crystals of free serpins can not be "docked" rigidly into proteases. An important consideration is the precise extent of insertion of the reactive center loop into the A-sheet. Insertion may provide the mechanism for straining the reactive center loop both at the time of docking with the protease and in the subsequent locking conformation[13] that stabilizes the complex.

Indeed, mutants in which the substitution predictably interferes with the insertion of the reactive center loop into the A-sheet are easily cleaved; for instance, P10-proline antithrombin[14]; this is unsurprising because prolines are not accommodated into non-edge strands of β-sheets because of their inability to form hydrogen bonds. However, prolines are less flexible than other residues.[15] Charged residues cannot be buried in the interior of a protein without neutralization of the charge. In the serpins, the even-numbered P residues have sidechains pointing into the protein. Thus, mutations of these "P-even" residues to charged sidechains would be expected to have greater effects than mutating the "P-odd" residues, the sidechains of which point into solution. For instance, the P12 Ala→Glu mutant of C1 inhibitor is a substrate rather than an inhibitor,[16] whereas the engineered variant P11 Ala→Glu of α_1-antitrypsin retains its inhibitory activity.[17]

A requirement for designing models of serpin-protease complexes is the definition of the extent of reactive center loop insertion that occurs upon complex formation. Here we present our grounds for building models in which it is inserted as far as P12.

Several lines of evidence show that insertion takes place at least as far as P14: first, the crystal structure of antithrombin (see Fig. 1d) implies that residues P14–P15 are inserted. A second line of evidence comes from experiments with peptide complexes: Schulze et al.[18,19] found that a synthetic peptide with the sequence of residues P1–P14 of α_1-antitrypsin could interact with this serpin resulting in the loss of inhibitory activity.[18]

There is evidence that insertion proceeds further in the protease-bound form than seen in uncleaved nonlatent conformations in the crystal structures: certain antibodies recognize complexed and cleaved antithrombin, but not its native form. These antibodies also recognize antithrombin when a synthetic 14-residue peptide is inserted into the A-sheet.[20] In the cleaved form it is known that the S→R transition has occurred. Therefore, the recognition of complexed antithrombin strongly suggests that the S→R transition has occurred in the complex. Conversely, the heparin pentasaccharide which binds

strongly to a defined site on native antithrombin *only binds weakly to* cleaved and latent antithrombin. A similarly weak bonding to antithrombin complexed to thrombin suggests that the complexed antithrombin is in the R rather than the S conformation.[21]

Several hinge region mutations which have a severe effect on the function of serpins as inhibitors help delineate more precisely the extent of insertion.

Mutations of residues in the region P10–P14 of the hinge region generally interfere with inhibitory activity (see Table II). The P8 Glu mutant of α_1-antitrypsin has normal function.[17] P10 Ser (antithrombin Cambridge II) loses some inhibitory activity towards thrombin, but not towards factor Xa.[22] In contrast, we have already mentioned that the P10 Pro mutant of antithrombin[15] loses inhibitory activity towards both thrombin and Factor Xa. The P10 Glu mutant of α_1-antitrypsin has a relatively minor effect,[18] whereas the P10 Pro mutant of α_1-antitrypsin has a severe effect on inhibitory activity.[12] The P10 Ala→Thr mutant of C1 inhibitor has lost inhibitory function, but this has been shown to result from polymerization of the serpin.[23]

Mutations beyond residue P14 include the P12 Ala→Glu mutant of C1 inhibitor[16] and P12 Thr (Hamilton) mutant of antithrombin[24] which are converted from inhibitors to substrates, and the P12 Glu α_1-antitrypsin which is predominantly a substrate.[17]

Our interpretation of these results is that the reactive center loop can be inserted as far as P12 with P10 in a tight turn and P8 exposed.

Previous Modeling of Serpin-Protease Complexes

The importance of the structure of the protease-bound form of serpins has, in the absence of an experimentally-determined structure, motivated several attempts to build models. However, previous modeling investigations have been based on insertion of the loop no further than P14. A model of the α_1-antitrypsin Pittsburgh-thrombin complex by Engh et al.,[25] shows the α_1-antitrypsin in the native conformation with insertion to P14. The loop is modeled into the reactive center cleft of thrombin. A model of the antithrombin-thrombin complex by Schreuder et al.,[7] shows an approximation to the rigid-body docking of thrombin to antithrombin based on the structure of uncomplexed uncleaved antithrombin. In a model of the antichymotrypsin-chymotrypsin complex also, the reactive center loop is inserted up to P14.[26] The serpin is in the native conformation. The residues in the reactive center loop (P'3–P3) were modeled as a canonical conformation based on the inhibitor residues in the turkey ovomucoid third domain-chymotrypsin complex.

None of these models shows any conformational change in the serpin, including in particular the S→R transition. We, therefore, concluded that models of intact serpin-protease complexes are more appropriately based on the assumption of loop insertion up to P12 with movement in strand s1C to allow this amount of insertion into the A-sheet.

The models of Engh et al. and Schreuder et al. contain intact serpins. In contrast, Wright and Scarsdale have modelled complexes of proteases with cleaved serpins[27] (see [28,29]).

TABLE III. Experimental Association Rates of Complexes Modeled

Complex	k_{ass} $M^{-1}\cdot s^{-1}$	Reference
α_1-antitrypsin-human neutrophil elastase	6.5×10^7	52
α_1-antitrypsin Pittsburgh-tryspin	2.7×10^6	52
α_1-antitrypsin Pittsburgh-thrombin	1.08×10^6	53
Antithrombin-thrombin (without heparin)	1.1×10^4	54

Design Features of Models Reported Here

We have modeled serpin-protease complexes with these properties:

- Insertion of the reactive center loop as far as P12, consistent with proper stereochemistry throughout the loop, including in particular the hinge region.
- Adoption of the canonical structure of the region around the scissile bond (P'3–P3), consistent with the crystal structures of complexes of small inhibitors and serine proteases, in which residues P'3–P3 of the inhibitory loop adopt similar structures.[30] Hubbard et al.,[31] showed that the r.m.s. deviations of the backbone atoms of these regions in protease-inhibitor complexes are no greater than 0.7 Å. We assume that the reactive center loops of the serpins in their protease complexes adopt this canonical conformation, and have imposed this as a constraint on the models.
- Capability to dock to all proteases that the serpin inhibits, without steric hindrance. In the cases of thrombin complexes, this is complicated by the presence of the two extra loops on the protease.

The basic challenge was to exhibit models in which the loop that interacts with the protease is both in a canonical conformation and in a position that allows complex formation. We found that the requirement of getting local structural details correct (i.e., an insertion to P12 and good stereochemistry of the loop) placed very severe constraints on the relative geometry of protease and inhibitor. Indeed, only one relative position of thrombin and antithrombin could be found. Table III lists the models we constructed and the experimentally-measured association constants of these protease-inhibitor pairs.

METHODS

Co-Ordinates and Calculations

The structures of human α_1-antitrypsin, antithrombin, and neutrophil elastase complexed with Methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethylketone, bovine trypsin complexed with porcine pancreatic secretory trypsin inhibitor and pancreatic trypsin inhibitor have been deposited in the Protein Data Bank.[32] (See Table I.) The co-ordinates of thrombin complexed with fibrinopeptide A (solved by Dr. W. Bode) were kindly supplied by Dr. S. Stone, and those of PAI-1 by Dr. E. Goldsmith.

Modeling, energy refinement and other calculations were done using QUANTA and CHARMm (Molecular Simulations, Inc., San Diego, CA) and progams of one of us.[34–35]

Modeling Procedures

The complex between α_1-antitrypsin Pittsburgh (P1 arginine) and thrombin has the most severe set of constraints to satisfy, to avoid steric hindrance from the extra surface loops on thrombin. (In Fig. 2a,b, the 149E loop is shown in pink and the 60F loop in brown.) Starting with the structure of cleaved α_1-antitrypsin, we mutated the P1 residue to an Arg to produce a molecule with the sequence of α_1-antitrypsin Pittsburgh. We then linked the P1 residue at the bottom of the A-sheet to the P'1 residue, applied constraints to fix the entire structure apart from residues 342–360 and minimized the energy to produce a stereochemically reasonable structure with the reactive center loop reconfigured to reform the peptide bond. The structure produced corresponds to the locking state (i.e., with partial insertion of the reactive center loop into the A-sheet causing separation of strands s3A and s5A) of α_1-antitrypsin, with insertion of strand four into the A-sheet down to P12, in a molecule with no breaks in the backbone.

To explore different relative positions of protease and inhibitor, we built a model containing two fragments: (1) the thrombin, plus a hexapeptide corresponding to residues P3–P'3 of the antitrypsin, with the mainchain set to the canonical conformation of the homologous hexapeptide of pancreatic secretory trypsin inhibitor from the crystal structure of its complex with trypsin; and (2) the α_1-antitrypsin (Pittsburgh) model with the P3–P'3 hexapeptide excised. We could then move the thrombin plus P3–P'3 hexapeptide independently from the rest of the serpin.

The fragments were moved manually to determine a position with no backbone clashes and few sidechain clashes. (The models, so produced, could subsequently be refined by conformational energy minimization.) Bad contacts were observed by identifying clashes visually and then applying distance monitors. As the fragments were moved, it was possible to observe the changes in distance between offending residues until a satisfactory solution was found. The 60D loop on thrombin imposed severe constraints on the position of the thrombin. A lesser factor (because of its apparent mobility as shown by its different conformations in thrombin-PPAK and thrombin-FPA) was the 149F loop of thrombin.

We found only one relative position of the two moieties in which there were no backbone clashes and the thrombin was in a position that allowed significant insertion of the reactive center loop into the A-sheet of the serpin. We then linked residue P4 to P3 using a program described by Lesk.[33] This program allows chain closure by a search in torsion angle space while constraining the deviations of the main chain conformational angles from their initial values. The same procedure was applied to link residues P'4 to P'3 (with movement allowed in residues P'9 and P'4).

This produced a complete model of α_1-antitrypsin docked to thrombin with no backbone clashes and with the reactive center loop in an acceptable conformation.

The whole model was then subjected to conformational energy minimization with the protein backbone constrained, until the energy converged. Then all constraints were removed and the structure minimized again. The resulting structure had a low (negative) conformational energy, indicating that it was sterically acceptable, and the Ramachandran plot showed most residues in allowed conformations. However, residues P5–P11 and P'5–P'10 were outside the allowed region for nonglycine residues.

Although we expected this region to be under strain, we deemed it important to determine how close to an ideal conformation could be achieved. Further minimization was carried out on the reactive center loop, with dihedral restraints applied to residues P5–P11 and P'5–P'10, and the P'3–P3 region constrained to maintain the canonical structure. The result was a loop with both a satisfactory Ramachandran plot and a low conformational energy.

This model was the basis for models of the complexes of α_1-antitrypsin Pittsburgh-trypsin and α_1-antitrypsin-elastase. For the elastase complex, the P1 residue (an Arg in α_1-antitrypsin-Pittsburgh) was converted back to a Met. We then superposed human neutrophil elastase and trypsin onto the thrombin moiety of the α_1-antitrypsin-thrombin complex, using a structure-alignment program.[34] The structures of elastase and trypsin used each contained an inhibitor; thus these calculations dealt with three complexes: our model of the α_1-antitrypsin-thrombin complex, the experimental structures of the trypsin-porcine pancreatic trypsin inhibitor (PSTI), and the elastase-methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethylketone complexes. Although the superpositions involved the protease moieties,

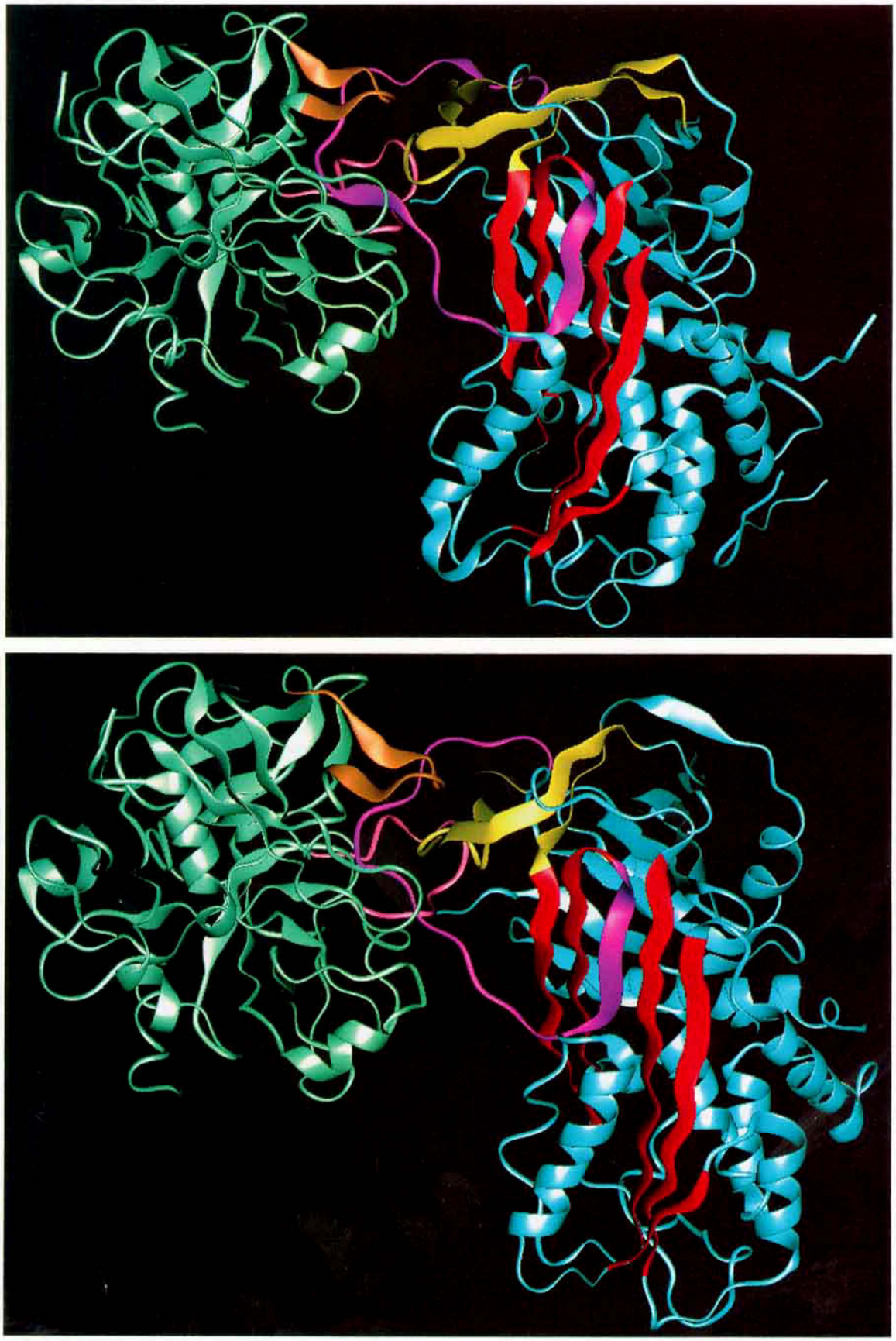

Fig. 2. Models of serpin-protease complexes. **a:** Antithrombin-thrombin. The protease is in light green and the serpin in light blue. In all parts of this figure, the A-sheet is red, the C-sheet yellow, and the reactive center loop is highlighted in purple from the top of the A-sheet to the end of s1C. In thrombin complexes, and 60F loop is in brown and the 149E loop in pink. **b:** α_1-antitrypsin Pittsburgh-thrombin. **c:** α_1-antitrypsin Pittsburgh-trypsin. **d:** α_1-antitrypsin-elastase.

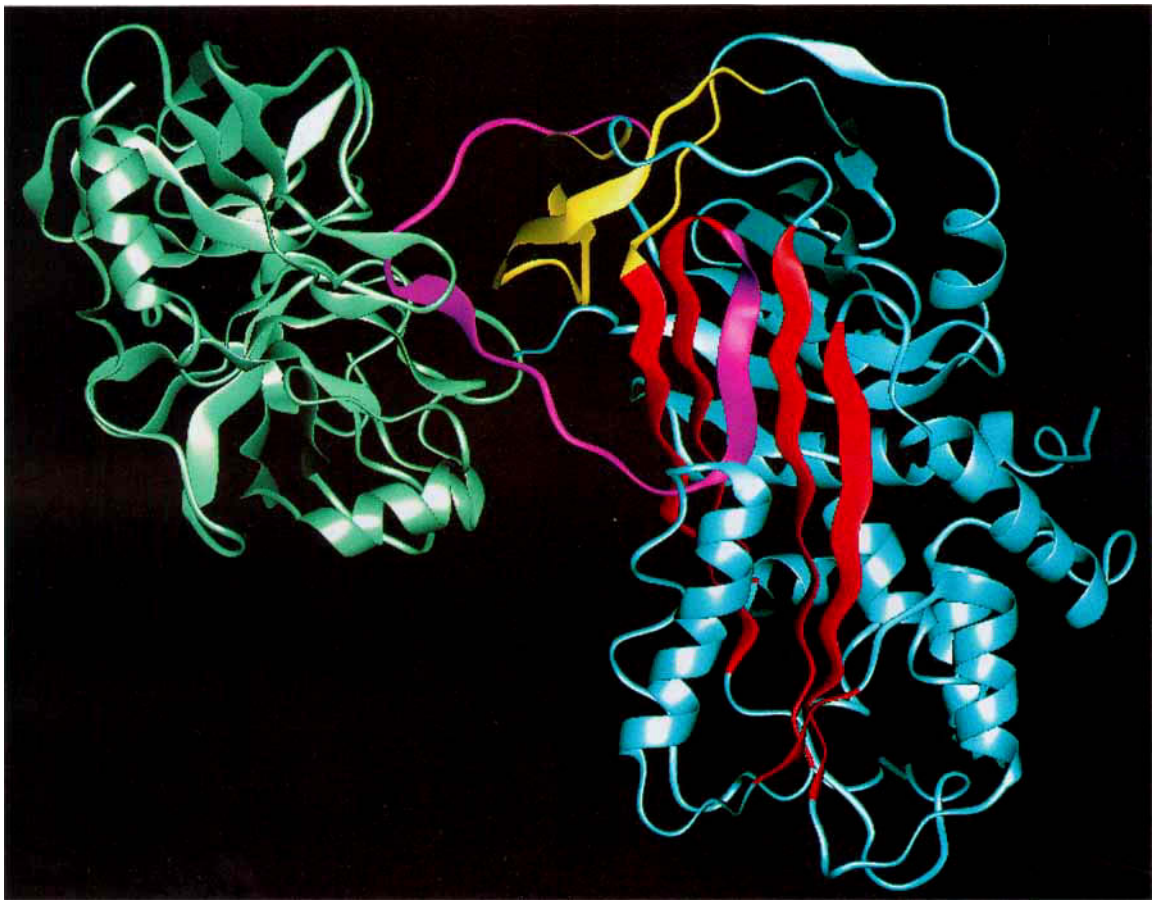

Fig. 2c.

they brought the P3–P'3 residues of the inhibitor, α_1-antitrypsin, into coincidence with P3–P'3 residues of the pancreatic secretory trypsin inhibitor in the trypsin-PSTI complex, or the P3–P1 residues in elastase complex (the small inhibitor does not contain residues corresponding to P'1–P'3).

Minimization was then carried out as before (first with the peptide backbone constrained and then without constraints). Dihedral constraints were then applied to the reactive center loop and the loop energy minimized.

The antithrombin-thrombin model was created in the same way as the α_1-antitrypsin-thrombin model.

RESULTS

Structures of the Models

Figure 2a–d shows the four models. In each, the protease is shown in green and the serpin in blue. The C-sheet is highlighted in yellow and the A-sheet in red. The reactive center loop (from the top of the A-sheet and including s1C) is purple. In thrombin, the 60F and the 149E loops are highlighted in brown and pink, respectively.

In all the complexes, the serpin is in the locking state with insertion of strand four into the A-sheet up to P12, with P11 the first residue coming out of the sheet. A tight turn is formed by residues P9–P11 (Fig. 3a–f). This strand then goes on to enter the cleft in the protease, with residues P3–P'3 in a canonical conformation. At the C-terminus of the reactive center loop strand, s1C is peeled away from the C-sheet as required for insertion of the reactive center loop into the A-sheet as far as P12. Insertion only as far as P14 would not require this change in the C-sheet, as demonstrated by previous model building (see above, and Wei et al.[12]).

In the models containing thrombin, the two extra loops are highlighted. Residues in these loops make hydrogen bonds to the antithrombin and α_1-antitrypsin.

Quality of the Models

The models have low conformational energy

The final converged conformational energies of the four models as calculated by CHARMm are in the range $-31{,}000$–$-39{,}000$ kcal·mol^{-1}, showing that there are no serious sterochemical defects; in particular, no steric clashes. In both models contain-

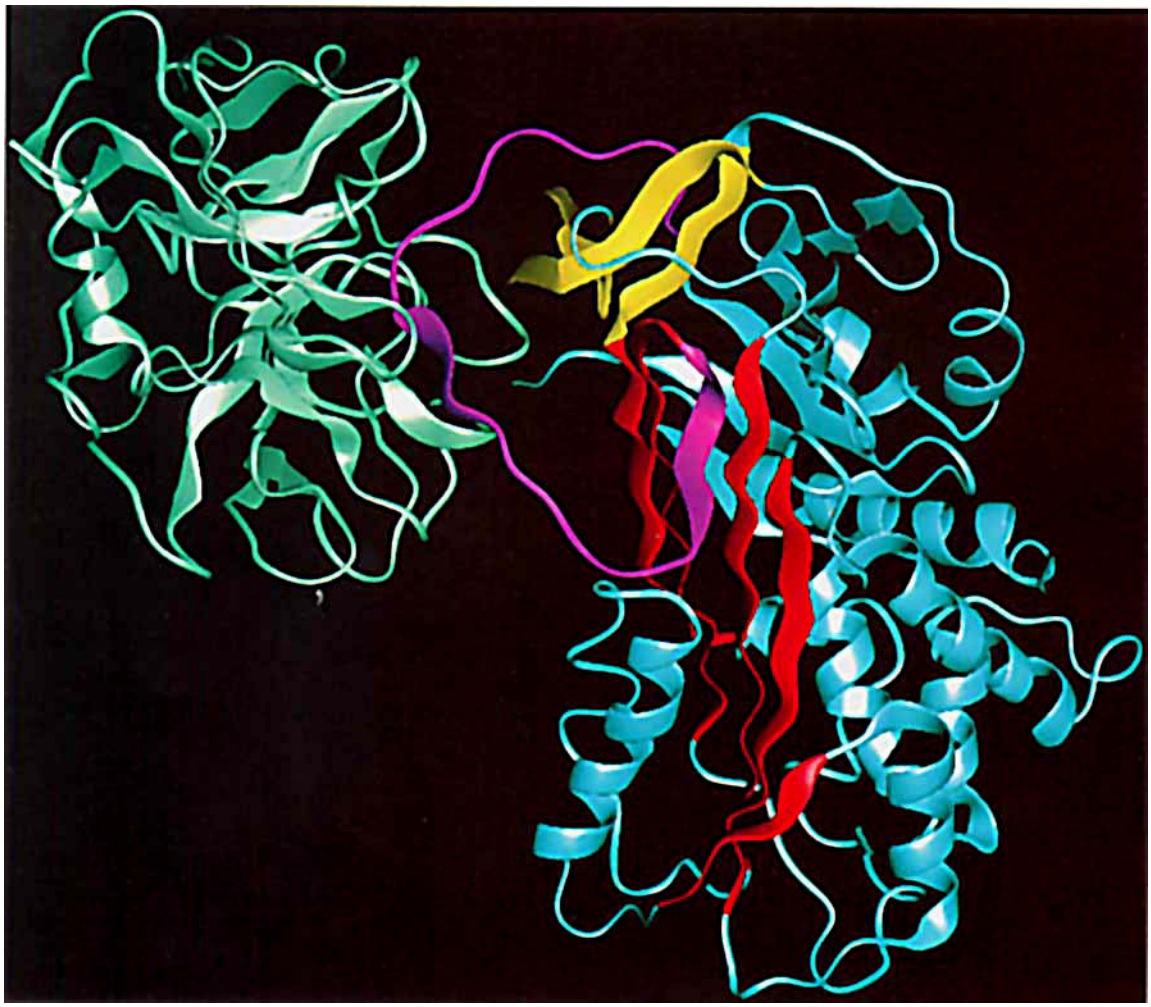

Fig. 2d.

ing thrombin, the free ends of the thrombin were constrained during minimization.

The models have allowed mainchain conformations

Residues 340–375 in α_1-antitrypsin and residues 375–410 in antithrombin comprise the reactive center loop of these molecules, plus a short surrounding region. Ramachandran plots of these regions for the four models show that all residues are in allowed conformations to within 20°. The values of the ω angles in this region are also satisfactory. Figure 3a–f shows the detail of the turn out of the A-sheet.

The models show extensive intermolecular hydrogen bonding

Table IV lists the hydrogen bonds formed between the inhibitor and protease.

The models show burial of hydrophobic surface similar in magnitude to, or greater than, known protease-inhibitor complexes

The accessible surface area[35] of the protease and relevant inhibitor were calculated separately and added together, and that of the complex subtracted to give the change in accessible surface area upon complex formation (Table V).

Conformational Changes Upon Complex Formation

Figure 4 shows the superpositions of the parent serpin structures onto the serpin moieties in the complexes. In order to measure the extent of structural change, we calculated superpositions of the inhibitors and enzymes in each complex onto their parent structures, and the r.m.s. and individual deviations of the corresponding atoms (see Table VIa). Backbone atoms (omitting oxygens) were used. Sev-

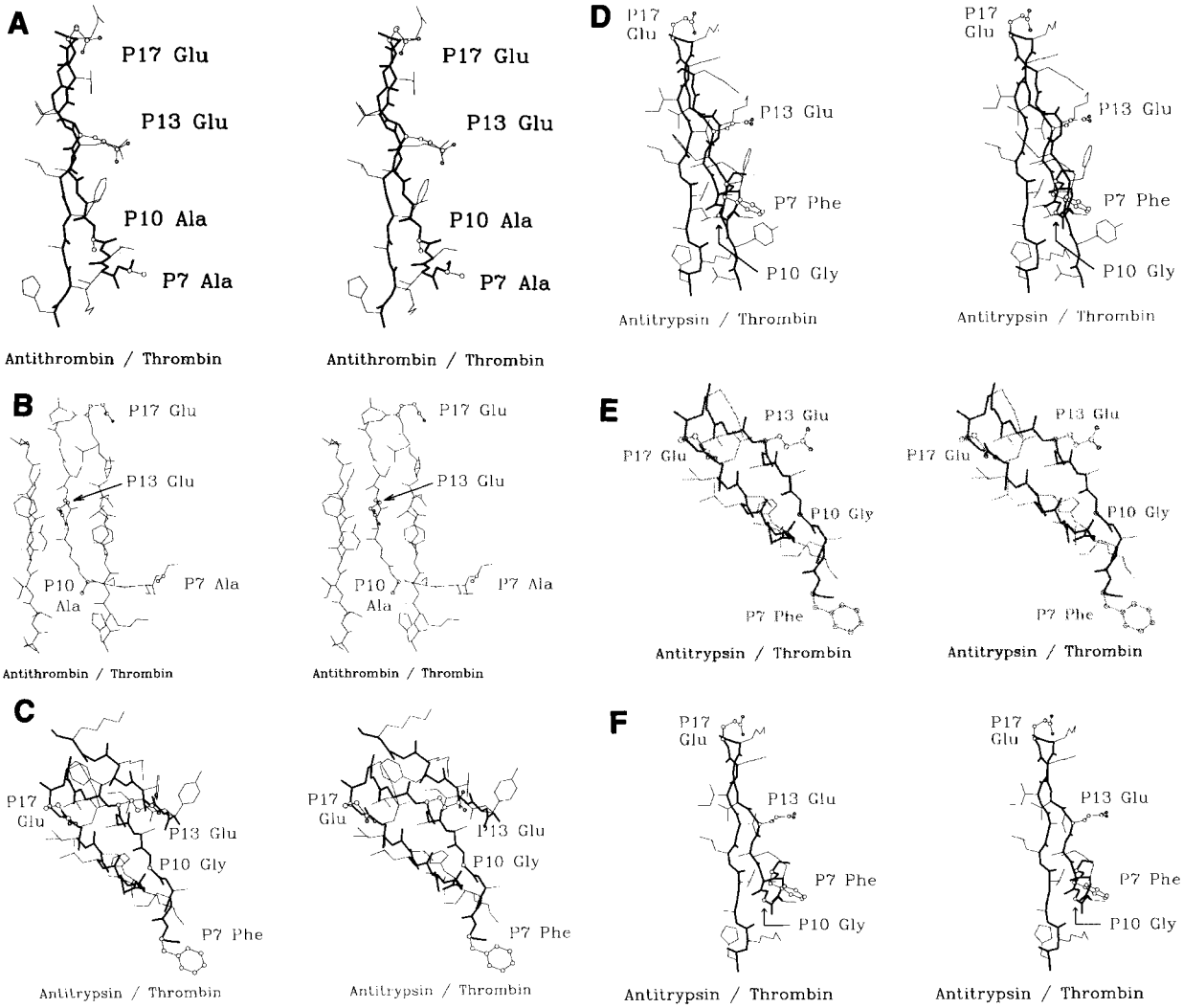

Fig. 3. The conformation of the reactive center loop in the models. **a,b:** Two representations of the reactive center loop coming out of the A-sheet in the antithrombin-thrombin model. The protein backbone is bold and most sidechains light; sidechains of residues P7, P10, P13, and P17 are shown in ball and stick representation. Figure 3a shows only s3A and s4A of the A-sheet with strand s4A (the reactive center loop) curling out of the page. Figure 3b shows three strands of the A-sheet—s3A, s4A, and s5A—with the reactive center loop, the middle strand, curling into the page. **c–f:** Four representations of the reactive center loop coming out of the A-sheet in the α_1-antitrypsin Pittsburgh-thrombin model. Figure 3c,d shows two different orientations of the turn out of the A-sheet with strands s3A, s4A, and s5A of the A-sheet present. Figure 3e,f shows two different orientations of the turn with only strand s3A and s4A present. The structure of this region is similar in other models of α_1-antitrypsin complexes.

eral of the complexes share a common moiety, and we calculated the differences in their mainchain conformation in the different models (see Table VIb). We also calculated deviations in atomic positions for the P3–P'3 regions of the complexes and the standard conformation as exemplified by the P3–P'3 regions of porcine pancreatic secretory inhibitor in the crystal structure of its trypsinogen complex (1tgs) (see Table VIc).

Comparison of crystal structure of free proteases and protease-inhibitor complexes show that proteases undergo little change upon binding inhibitor. Kaslik et al.[36] suggest that proteases may change conformation in substrate complexes, and that such changes may occur in serpin-protease complexes. This is based on the change in cleavage kinetics of a mutant rat trypsin upon binding to α_1-antitrypsin. However, the nature and magnitude of these changes are unknown.

The major structural changes in the serpins, relative to the starting conformations on which the models are based, were these: in the complexes constructed from cleaved α_1-antitrypsin, the peptide bond has been reformed to produce a single continuous chain. In all four complexes, the reactive center loop is inserted into the A-sheet up to P12, strand s1C is pulled out of the C-sheet, and there is a conformational change in the C-terminus of the serpin

TABLE IV. Protease-Inhibitor Hydrogen Bonds in Modeled Serpin-Protease Complexes

(a) Antithrombin-thrombin

Inhibitor				Protease
243Asp	O	...	N	148Trp
243Asp	OD2	...	N	149AAla
387Lys	O	...	NZ	224Lys
389Val	N	...	OE1	217Glu
391Ala	N	...	O	216Gly
391Ala	O	...	N	216Gly
393Arg	N	...	O	214Ser
393Arg	O	...	OG	195Ser
393Arg	NE	...	O	219Gly
393Arg	NE	...	SG	220Cys
393Arg	NH1	...	OD1	189Asp
393Arg	NH1	...	O	219Gly
393Arg	NH2	...	OD2	189Asp
393Arg	NH2	...	O	190Ala
394Ser	O	...	NZ	60FLys
394Ser	OG	...	NZ	60FLys
394Ser	OG	...	NE2	57His
395Leu	N	...	O	41Leu
399Arg	NH1	...	OE1	192Glu
432Lys	NZ	...	OE1	192Glu

(b) α_1-antitrypsin Pittsburgh-thrombin

Inhibitor				Protease
205Glu	OE1	...	NE1	60DTrp
211Asp	OD2	...	N	149AAla
212Gln	O	...	NZ	149ELys
212Gln	OE1	...	ND2	149BAsn
214Thr	OG1	...	O	146Glu
217Lys	NZ	...	O	60DTrp
356Ile	N	...	O	216Gly
356Ile	O	...	N	216Gly
358Arg	N	...	O	214Ser
358Arg	O	...	N	193Gly
358Arg	O	...	N	195Ser
358Arg	NE	...	O	216Gly
358Arg	NH1	...	OD1	189Asp
358Arg	NH1	...	O	219Gly
358Arg	NH2	...	OD1	189Asp
358Arg	NH2	...	O	190Ala
359Ser	O	...	NZ	60FLys
359Ser	OG	...	NZ	60FLys
394Lys	NZ	...	OE1	192Glu
394Lys	OXT	...	N	219Gly

(c) α_1-antitrypsin Pittsburgh-trypsin

Inhibitor				Protease
214Thr	OG1	...	OG1	149Thr
354Glu	OE1	...	HZ1	224Lys
354Glu	OE2	...	HZ2	224Lys
356Ile	N	...	O	216Gly
356Ile	O	...	N	216Gly
357Pro	O	...	NE2	192Gln
358Arg	N	...	O	214Ser
358Arg	N	...	OG	195Ser
358Arg	N	...	NE2	57His
358Arg	O	...	N	194Asp
358Arg	O	...	N	193Gly
358Arg	O	...	N	195Ser

(continued)

TABLE IV. Protease-Inhibitor Hydrogen Bonds in Modeled Serpin-Protease Complexes *(continued)*

(c) α_1-antitrypsin Pittsburgh-trypsin *(continued)*

Inhibitor				Protease
358Arg	NH1	...	OD1	189Asp
358Arg	NH1	...	O	219Gly
358Arg	NH2	...	OG	190Ser
360Ile	N	...	O	41Phe
360Ile	O	...	OH	39Tyr
394Lys	NZ	...	OE1	192Gln
394Ser	O	...	NZ	60FLys
394Lys	OXT	...	NE2	192Gln
394Lys	OXT	...	N	219Gly

(d) α_1-antitrypsin Pittsburgh-human neutrophil elastase

Inhibitor				Protease
214Thr	OG1	...	O	145Gly
354Glu	O	...	N	218Gly
356Ile	N	...	O	216Val
356Ile	O	...	N	216Val
358Met	N	...	O	214Ser
358Met	N	...	OG	195Ser
358Met	O	...	N	195Ser
358Met	O	...	N	194Asp
358Met	O	...	N	193Gly
359Ser	OG	...	OG	195Ser
359Ser	OG	...	SG	42Cys
359Ser	OG	...	NE2	57His
360Ile	N	...	O	41Phe
363Glu	OE1	...	NE	36Arg
394Arg	N	...	O	218Gly

TABLE V. Change in Accessible Surface Area Upon Complex Formation

Complex	Δ (accessible surface area)/Å^2
Antithrombin-thrombin	−2249
α_1-antitrypsin Pittsburgh-thrombin	−2408
α_1-antitrypsin Pittsburgh-trypsin	−1763
α_1-antitrypsin-elastase	−1865

arising from interactions between the protease and the C-terminal lysine of the serpin. Outside these regions the changes are small, and attributable to changes in the surface loops of the serpin. If residues 383–407 (348–369) and 429–432 (391–394) in antithrombin, or 348–367 and 391–394 in α_1-antitrypsin, are removed, the r.m.s. deviations between the serpin in the modeled complex and the parent structure are in the range 1.2–1.3 Å. We further note that the conformations of the α_1-antitrypsin in the three models containing it did not vary significantly in complex with the different proteases (see Table VIc).

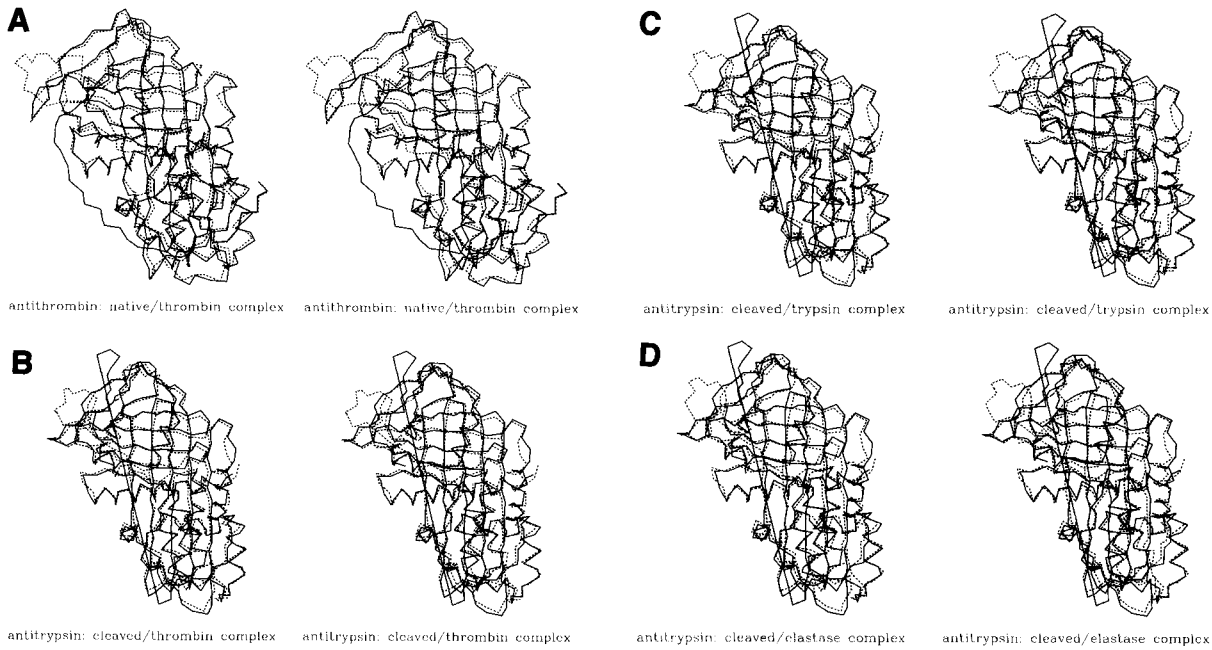

Fig. 4. Superpositions of serpin conformations in models of complexes with the parent structures. **a:** Antithrombin from the antithrombin-thrombin model. The parent (latent) structure is shown in bold and the model in broken lines. **b:** α_1-antitrypsin from the antitrypsin Pittsburgh-thrombin model. The parent antitrypsin structure (7api) is shown in bold and the model in broken lines. In parts b–d, the cleaved ends (P'1–P1) are joined with a straight line. **c:** α_1-antitrypsin from the α_1-antitrypsin Pittsburgh-trypsin model. The parent α_1-antitrypsin structure (7api) is shown in bold and the model in broken lines. **d:** α_1-antitrypsin from the α_1-antitrypsin-elastase model. The parent α_1-antitrypsin structure (7api) is shown in bold and the model in broken lines.

DISCUSSION

Structural Features of the Models

In this section, we examine the modeled complexes in terms of properties of protease-inhibitor complexes of known structure.

Accessible surface area buried in the interfaces

Janin and Chothia[37] have emphasized the importance of burial of protein surface for the stability of protease-inhibitor complexes. For protease-inhibitor complexes of known structure, the amount of surface area buried in the interface ranges from 1,250 Å^2 to 1,700 Å^2, except for the thrombin-hirudin complex.

In our models, the surface area buried is above this range. For the α_1-antitrypsin-elastase and α_1-antitryspin (Pittsburgh)-trypsin models, the area buried in the interface (1,800 Å^2) is comparable to that of trypsinogen-pancreatic secretory inhibitor (1,750 Å^2). The two thrombin complexes have a significantly higher buried surface area (2,250 and 2,400 Å^2) than any protease-inhibitor complex of known structure except thrombin-hirudin (3298 Å^2). The buried surface area at the serpin-protease interface is likely to make an important contribution to the strong protease-serpin interaction.

Hydrogen bonding

The number of hydrogen bonds in protease-inhibitor complexes of known structure ranges from 8–13, with an average of 10.[37] Our modeled complexes have more intermolecular hydrogen bonds, ranging from 15 (in α_1-antitrypsin-elastase) to 21 (in antithrombin-thrombin). Most of these are clustered around the reactive center cleft. There are some additional interactions, notably with the 149E loop of thrombin. The increased number of hydrogen bonds may account for the greater stability of serpin-protease complexes and their irreversibility when complexes are formed. It is possible that the effect does not arise directly from the energetics of hydrogen bond formation, but by the effect of the hydrogen bonds on the details of the conformation at the interface.[38,39]

Although in general the serpin-protease interactions are quite similar in the four complexes, the subtle differences which the serpins have evolved to achieve specificity in their protease interaction may involve differences in the hydrogen bonding pattern. A particular hydrogen bond that appears critical in the binding of the reactive center loop into the protease cleft involves protease residue 192. In thrombin, the residue at this position is Glu; in trypsin, Gln and in human neutrophil elastase, Phe. In the antithrombin-thrombin model, within the inhibitor 326 Glu (294) interacts with 386 Thr (351) and 388 Val (353). The latter two residues are just past the turn out of the A-sheet (P8 and P6). These interactions are not present in any of the α_1-antitrypsin models. This suggests that mutants at this position

TABLE VI. Structural Differences Between Parent Structures and Models, and Between Models

(a) Comparisons of mainchain conformations of serpins and proteases between the parent structures and the modeled complexes. The r.m.s. deviations of the entire structures are dominated by a few regions that show large conformational changes. The selected substructures include most of the structure; the regions deleted are 383–407 (348–369) in antithrombin and 348–367 and 391–394 in α_1-antitrypsin

	r.m.s. deviation from parent/Å	
Subunit	Entire structure	Selected substructure
Antithrombin	6.1	1.2
Thrombin	1.1	
α_1-antitrypsin Pittsburgh	6.1	1.2
Thrombin	1.1	
α_1-antitrypsin Pittsburgh	6.1	1.2
Trypsin	0.6	
α_1-antitrypsin	6.1	1.3
Elastase	0.9	

(b) Comparison of the mainchain conformations of residues of P3–P'3 of the reactive center loop of the serpin in each model with that of the hexapeptide of conserved conformation of porcine pancreatic secretory trypsin inhibitor (PDB code 1tgs)

Complex	r.m.s. deviation/Å
Antithrombin-thrombin	0.85
α_1-antitrypsin Pittsburgh-thrombin	0.51
α_1-antitrypsin Pittsburgh-trypsin	0.42
α_1-antitrypsin-elastase	0.58

(c) Differences between mainchain conformations of the common moiety (shown in boldface) of different modeled complexes

Complexes compared		r.m.s. deviation/Å
Antithrombin-**thrombin**	α_1-antitrypsin Pittsburgh-**thrombin**	0.6
α_1-**antitrypsin Pittsburgh**-trypsin	α_1-**antitrypsin Pittsburgh**-thrombin	0.2
α_1-**antitrypsin Pittsburgh**-thrombin	α_1-**antitrypsin**-elastase	0.6
α_1-**antitrypsin**-elastase	α_1-**antitrypsin Pittsburgh**-tryspin	0.5

would provide interesting results. On the basis of our model, we predict that mutating 326 Glu (294) to Ala would affect the stability of the complex.

The Mechanism by Which Serpin Mutants Affect Function

The general principle by which we interpret the functional properties of mutants is that, to function as an inhibitor, the reactive center loop must be held rigid or even under strain, else the molecule becomes a substrate. Our working hypothesis is that serpins achieve this regidity by inserting part of the reactive center loop into the A-sheet.[12]

Perturbation of the critical mobile domains of the serpins can also promote spontaneous transformations to the inserted-loop conformations of the latent form, or to an intermediate form, comparable to the locking conformation present in the serpin-protease complex. The evidence for such a locking conformation has recently been summarized by Stein and Carrell.[13] They showed that the mutations in 97 dysfunctional human variants are clustered in the critical mobile domains of the molecule, including the proximal and distal hinges of the reactive loop. Evidence that spontaneous conformational changes are a common feature of the mobile-domain mutants is provided by studies of antithrombin[20,40] and C1-inhibitor.[41] Some of the mutations in these mobile domains predispose the molecules to polymerization.

Specific Mutants

Mutants of P14 and P12

In the models, both the P14 and P12 residues are inserted into the A-sheet, with their sidechains pointing into the structure. Mutations at these positions to charged sidechains should prevent their insertion and affect inhibition. α_1-Antitrypsin P14 Arg and P12 Glu[17] have lost most, but not all, of their inhibitory activity (P14 Arg retains <1% inhibitory activity; P12 Glu retains ~25% inhibitory

activity). The reason for the small amount of residual inhibitory activity is not clear, but modeling has shown that the polar residues can insert their proximal methylene groups into the sheet, with the distal charged atoms accessible to water.

Mutants at P10

In the α_2-antitrypsin models, the P10 residue is located on the turn as the loop leaves the A-sheet. In α_1-antitrypsin, this residue is a Gly. The conformation of this residue is just outside the allowed region for non-glycine residues. The P10 Pro mutant of α_1-antitrypsin is predominantly a substrate[10] (see Table II). Replacement of the Gly with a Pro may prevent the loop from making this turn easily, precluding the region as a whole from taking up the conformation proposed in the models. Interestingly, a P10 Glu mutant of α_1-antitrypsin does not have the effect of the P10 Pro; rather it seems to introduce only a slight destabilization: the complex breaks down after long periods of time (~10 hours).[17] These results suggest that the P10 residue is not buried, but that its conformation is critical. Further support for this comes from two variants of C1-inhibitor, where P10 Ala is replaced by Val or Thr, with loss of inhibitory function.

C-sheet mutants

Strand s1C together with the peptide loop linking it to strand s4B acts as a distal bridge for movement of the reactive center loop.[12] The critical function of this distal hinge, in keeping with our model, is indicated by the number of natural mutations within this region that result in loss of inhibitory activity. Stein and Carrell[13] list 12 such variants: eight variants of antithrombin that result in thrombotic diseases, three of C1-inhibitor resulting in the allergic disease angioedema, and one of α_1-antitrypsin resulting in emphysema. The interpretation of some of the mutations is straightforward as in the replacement of Pro369 at the s1C–s4B turn by Thr or Leu. Others are more subtle, such as the replacement of 366 Ala by Thr in α_1-antitrypsin or, at the same position Val by Met in C1-inhibitor. Eldering et al.,[41] have shown that such mutations in s1C produce changed antigenic properties, consistent with partial insertion of the reactive-center loop into the A-sheet. Their findings also support the exposure of a new antigenic site present in the latent and complexed forms, but not in the reactive-loop-cleaved serpins. The conclusion is that mutations which disrupt the binding of s1C result in a conformation either with properties of the locking conformation, or with properties of the latent form.

The consequences of the change in conformation in the mutants of the mobile domains, and of the distal hinge mutants in particular, is a loss of inhibitory activity and an increased intracellular turnover complicated by polymer formation.

C-terminal mutants

Mutants around the C-terminal region of antithrombin (e.g., antithrombin Budapest 429 (391) Pro→Leu[42] result in decreased inhibitory activity. In our models, the C-terminus is at the interface between protease and inhibitor, forming intermolecular hydrogen bonds. Disruption of this interaction could weaken affinity through conformational change in the C-terminus, with consequent distortion of the neighboring hinge region.

CONCLUSIONS

We have reported the models of four serpin-protease complexes: antithrombin-thrombin, α_1-antitrypsin (358 Met→Arg)-thrombin, α_1-antitrypsin (358 Met→Arg)-trypsin, and α_1-antitrypsin-elastase. We have shown it to be stereochemically possible for the reactive center loop to be inserted into the A-sheet as far as residue P12 in uncleaved serpins, and that such insertion produces a structure that can be docked into active sites of the proteases.

The models make it possible for us to analyze the conformational changes that might occur on complex formation. As in protease-inhibitor complexes observed crystallographically, the protease undergoes minimal conformational changes. In contrast, the starting conformation of antithrombin observed in the crystal structure could not dock rigidly to thrombin. Conformational change in the serpin is consistent with the observations that antibodies bind cleaved and complexed serpins but not the native (S-state) and, in contrast, high-affinity heparin binds tightly to the native form, but not to cleaved and complexed.

The major structural changes required to create the protease-bound form were: (1) The reactive center loop must change conformation from that of the native antithrombin structure. (2) The strand s1C must be pulled away.[12] In our models, strand s1C is not pulled out as far as in the latent forms of PAI-1 and antithrombin, but there is a gap in the C-sheet. This is consistent with the observation that the complexed serpin shares a common antigenic domain with the conformationally-modified s1C mutants. In addition, Wardell et al. (manuscript in preparation) have noted that lipoprotein receptor protein binds complexed and latent antithrombin, but not native or cleaved antithrombin. Therefore, an area must be exposed in the latent form which is not exposed in the cleaved form. The reactive center loop is not exposed in our models of the complexed form (it is bound into the cleft of the protease). In our model, strand s1C is pulled out and it is, therefore, a candidate region for the binding of LRP. (This maybe relevant to the formation of polymers by mutant serpins: there is now evidence that polymerization involves interaction of the P3–P7 portion of the reac-

tive loop of one molecule with the vacated s1C position of the next).

Significant structural features of the models include the following:

1. The reactive center loop can be inserted into the A-sheet as far as P12 while maintaining proper stereochemistry.

2. The conformation of the serpin in the complex is intermediate between the two uncleaved states observed in the crystal structure of antithrombin.[6]

3. Uniqueness of the relative position of the inhibitor and protease. The strict constraints in the design of the models—notably the insertion of the reactive center loop as far as P12—made it possible to generate models with essentially only a single stereochemically satisfactory relative geometry of inhibitor and protease. The interaction with thrombin appears to determine the relative geometry of the protease and inhibitor to within narrow limits. A lesser degree of insertion—for instance, only as far as P14—does not constrain so tightly the relative position of inhibitor and protease.

4. Nature of the protease-inhibitor interaction. The serpin and protease are held together by van der Waals and hydrogen-bonding interactions and the burial of surface area in the interface. The main interactions are between the reactive center loop and the protease. However, in the models of thrombin-serpin complexes, the 149E loop also interacts with the serpin. In all four models, the C-terminal lysine of the inhibitor is involved in hydrogen bonding to the protease.

5. The structures are consistent with the idea that in *normal* serpins, the reactive center loop is inserted into the A-sheet to the point of rigidity, as suggested by the observations that mutants that interfere with the insertion convert the serpin from an inhibitor to a substrate (see above). These mutants are at positions in which the sidechains point into the molecule, or that affect the conformation of the tight turn coming out of the sheet. Other mutants, at positions in which the sidechains are predicted to be on the surface, do not affect activity.

6. We suggest, in conclusion, that our models are as credible as possible in the absence of direct experimental test. Internal stereochemical consistency cannot in itself prove a model, but its absence can disprove one. The models built are free of steric clashes, as shown by the low conformational energies calculated by CHARMm, and the portions rebuilt from the parent structures have allowed mainchain conformations as shown by the Ramachandran plots and the values of the ω angles. These models should therefore provide a basis for discussing properties of mutants and guiding further experimental work.

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