Intermolecular Interactions between Protein C Inhibitor and Coagulation Proteases†

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ABSTRACT: Protein C inhibitor (PCI) inhibits multiple plasma serine proteases. To determine which residues contribute to its specificity of inhibition, 19 mutations in the reactive site loop of PCI (from Thr_{352} to Arg_{357}) were generated and assayed with thrombin, activated protein C (APC), and factor Xa. To identify the intermolecular interactions responsible for these kinetics, a molecular model of PCI was generated using α_1-protease inhibitor and ovalbumin as templates. This model of PCI was docked with thrombin, followed by extensive energy minimization, to determine a lowest energy complex. The resulting docked complex was used as a template to form molecular models of PCI−APC and PCI−factor Xa complexes. The best inhibitors of thrombin contained Pro or Gly at the P2 position in place of Phe_{353}, with 2- and 7-fold increases in activity, respectively. These substitutions reduced steric interactions with the 60-insertion loop unique to thrombin. The best inhibitors of APC and factor Xa contained Arg at the P3 position in place of Thr_{352}, with 2- and 5-fold increases in inhibition rates, respectively. The molecular model predicts that Arg in this position could form a salt bridge with Glu_{217} of each protease. Changing Arg_{357} at the P3′ position had little effect on protease inhibition, consistent with the observation in the model that this residue points toward the body of PCI, forming a salt bridge with Glu_{220}. Given its broad specificity of inhibition, PCI has proven very useful in understanding the nature of serpin−protease interactions using multiple mutations in a serpin assayed with multiple proteases.

The coagulation cascade is regulated in part by several plasma serine protease inhibitors (serpins) including antithrombin III (AT)[1] and heparin cofactor II (HCII). Serpins have reactive site loops that insert into the active site cleft of a protease. The protease then cleaves the serpin between the P1−P1′ bond, resulting in the formation of a covalent intermediate. Protein C inhibitor (PCI) is a heparin-binding serpin which physiologically inhibits activated protein C (APC) (Marlar et al., 1993). The P3 through P3′ residues in the reactive site loop of PCI are Thr_{352}-Phe_{353}-Arg_{354}-Ser_{355}-Ala_{356}-Arg_{357}, and the protease cleaves between the Arg_{354}−Ser_{355} bond. PCI inhibits several additional serine proteases *in vitro* including thrombin, factors Xa and XIa, urokinase, tissue plasminogen activator, prostate specific antigen, acrosin, kallikrein, trypsin, and chymotrypsin (Espana et al., 1989; Meijers et al., 1988; Pratt et al., 1989; Suzuki et al., 1983). Given its broad range of specificity, PCI is a good candidate for studying the intermolecular interactions between serpins and serine proteases.

Serine proteases comprise a large family of structurally homologous proteins whose main differences occur in loops termed variable regions (Greer, 1990). Thrombin has been cocrystallized with the small inhibitor D-Phe-Pro-Arg chloromethyl ketone (PPACK) (Bode et al., 1992) and the leech thrombin inhibitor hirudin (Rydel & Tulinsky, 1991) but not with a serpin. The serine protease trypsin has been crystallized with two macromolecular Kunitz-type inhibitors, bovine pancreatic trypsin inhibitor (BPTI) and soybean trypsin inhibitor (STI) (Janin & Chothia, 1976; Sweet et al., 1974). These crystal structures have defined the interactions between small inhibitors and proteases; however, it appears that small inhibitors and substrates do not bind in the active site clefts of serine proteases with the same interactions as macromolecular inhibitors and substrates (Stubbs & Bode, 1993).

To determine which residues are involved in the formation of a serpin−protease complex, many mutations have been engineered into both serine proteases and serpins. In thrombin, deletion of residues in loops flanking the active site results in decreased activity with antithrombin (Le Bonniec et al., 1992, 1993). In APC and thrombin mutagenesis of Glu_{217} to Gln results in increased inhibition by AT and Kunitz-type inhibitors (Guinto et al., 1994; Rezaie & Esmon, 1993). These changes also altered the proteases' preferences for P3 and P3′ residues in peptide substrates and activities with physiological substrates. Mutations in serpins have focused on the reactive site loop residues, primarily P2, P1, and P1′ (Derechin et al., 1990; Eldering et al., 1992; Heeb et al., 1990; Lane et al., 1991; Schechter et al., 1993), and on residues P10−P14, which partially insert into the β-sheets of the body of the serpin (Hopkins et al., 1993; Lane et al., 1991). These variant proteins have demonstrated the remarkable specificity of most enzymes for their substrates;

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; PCI, protein C inhibitor; AT, antithrombin; HCII, heparin cofactor II; IIa, thrombin.

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however, individual residues in contact between the protease and serpin have not been identified.

A crystal structure of a protease−serpin complex is the best tool in determining important contact points. Lacking this, a molecular model of a complex between thrombin and PCI was constructed. Similar docked models were generated for APC and factor Xa to test this model with multiple proteases. Previously, we have engineered reactive site mutations in the P2, P1, and P1′ residues of the reactive site of PCI and assayed them with several proteases, including thrombin, APC, and factor Xa (Cooper & Church, 1995; Phillips et al., 1994). In this paper we present kinetic data on reactive site mutations in the P3 and P3′ positions of PCI and correlate these kinetic data to molecular models of the docked complexes.

MATERIALS AND METHODS

Design and Expression of PCI Mutants. PCI mutants were generated using site-directed mutagenesis as described previously (Cooper & Church, 1995; Phillips et al., 1994). P3 and P3′ mutations were introduced with separate oligonucleotides containing degenerate codons. To address the role of charged amino acids in the reactive site loop, neutral (Gly) and positively (Arg, Lys) and negatively (Glu) charged amino acids were substituted for the P3 Thr_{352}. A similar approach was used at the P3′ position with Arg_{357} being replaced with His, Ala, Asp, and Pro. Recombinant proteins were expressed and their concentrations measured by ELISA as described previously (Cooper & Church, 1995; Phillips et al., 1994).

Protease Inhibition Assays. α-Thrombin was prepared as detailed previously (Church & Whinna, 1986). Factor X was purchased (Enzyme Research Laboratories) and activated as described previously (Monroe et al., 1988). APC was purchased (Haematologic Technologies Inc.). Assays were performed at room temperature in buffer containing 20 mM HEPES, 150 mM NaCl, 0.1% poly(ethylene glycol), 0.02% azide, and 2 mg/mL BSA at pH 7.4. Chromogenic substrates for the proteases were Lys-(Cbo)-Pro-Arg-*p*-nitroanilide (Spectrozyme PCa, American Diagnostica) for APC, tosyl-Gly-Pro-Arg-*p*-nitroanilide (Chromozym TH, Boehringer-Mannheim) for thrombin, and MeO-CO-D-CHG-Gly-Arg-*p*-nitroanilide (Spectrozyme fXa, American Diagnostica) for factor Xa. Unbleached crude heparin was obtained from Diosynth (Oss, The Netherlands). Protein concentrations for the reactions shown in Figure 2 were 0.5 nM thrombin, 5 nM rPCI, and 1 μg/mL heparin; 1 nM APC, 10 nM rPCI, and 10 μg/mL heparin; and 1 nM factor Xa, 50 nM rPCI, and 1 μg/mL heparin. Second-order rate constants of inhibition were calculated using the equation $k'/[I] = (-ln\ a)/t[I]$, where a is residual protease activity relative to an uninhibited control, t is time, and [I] is the serpin concentration. Each reaction was performed two to four times in triplicate, and averages were calculated. Relative k′/[I] values were calculated by dividing the k′/[I] value for a mutant protein by the value for wild-type recombinant PCI (rPCI) under the same assay conditions.

Molecular Modeling of PCI. The X-ray crystal structures of α_1-antitrypsin (Engh et al., 1989), turkey ovomucoid third domain (Fujinaga et al., 1987), ovalbumin (Stein et al., 1990), and AT (Carrell et al., 1994) were obtained from the Brookhaven Protein Data Bank. The sequence of PCI (Suzuki et al., 1987) was obtained from the Protein Identification Resource data bank. Molecular models were generated using the Homology, Biopolymer, and Discovery modules of Insight II (version 2.3.0; Biosym Technologies, Inc.).

The molecular model of PCI was generated in a manner similar to that for HCII (Whinna & Church, 1994). The program Homology was used to align the protein sequence of PCI with ovalbumin and α_1-antitrypsin. The functionally inactive "reactive site loop" of ovalbumin was superimposed onto that of turkey ovomucoid. The region of the loop from amino acids 348 to 352 was superimposed on the corresponding region of the intact AT crystal structure (Carrell et al., 1994). The reactive site loop was manipulated to fit into the active site cleft of the crystal structure of thrombin. For PCI to be active, we assumed that the reactive site loop must be able to attain a conformation that would allow insertion into the relatively inaccessible active site of thrombin.

Energy minimizations were performed by first allowing only the residues 335−344 and the two introduced loops to move. PCI was minimized by steepest descent until the maximum derivative was less than 5 kcal/(mol·Å) and then by conjugate gradient to 0.1 kcal/(mol·Å). The residues partially inserting into the parallel β-sheets, 335−344, were fixed, the model of PCI was covered with a 10 Å shell of water, and steepest descent minimizations were performed to 2 kcal/(mol·Å). Finally, conjugate gradient minimization was performed with a 10 Å shell of water, Morse terms, cross terms, and the region 335−344 held constant, until a maximum derivative of less than 0.1 kcal/(mol·Å) was achieved.

Molecular Modeling of the Protease Domains of APC and Factor Xa. The three-dimensional coordinates of PPACK-thrombin were generously provided by Dr. Wolfram Bode (Bode et al., 1992). The X-ray crystal structure of trypsin (Marquart et al., 1983) was obtained from the Brookhaven Protein Data Bank. The sequences of protein C (Foster et al., 1985) and factor X (Kaul et al., 1986) were obtained from the Protein Identification Resource data bank.

Only the active site and surrounding domains of protein C (APC) and factor X (factor Xa) were modeled. The Align Structure command in Homology was used to align the structures and sequences of the X-ray crystal structures of thrombin and trypsin. This template was then used to assign coordinates to generate models of APC and factor Xa. Most sequence variability occurs in loops, and these were modeled using the Loops command in the program Homology. The carboxy terminus of thrombin is α-helical, and secondary structure predictions for protein C suggest that its α-helix extends four more residues. The remaining seven residues of APC were generated by the EndRepair command. A similar procedure was performed for factor Xa. The appropriate disulfide bonds were formed using Biopolymer with factor Xa have one more disulfide than thrombin and APC.

Energy minimizations of APC and factor Xa were both performed using the cvff force field in Discover. Steepest descent minimizations *in vacuo* were performed with the backbone first tethered and then free each time to a maximum derivative of 10 kcal/(mol·Å). The molecules were then surrounded by a shell of water and minimized with steepest descents to 50 kcal/(mol·Å), followed by conjugate

gradients to 1 kcal/(mol·Å). At this point cross and Morse terms were added and the molecules minimized to 0.1 kcal/(mol·Å).

Formation of a PCI−Thrombin Complex. The reactive site P1 side chain of PCI, Arg_{354}, was superimposed onto the Arg residue of PPACK in the crystal structure of PPACK-thrombin. PPACK was then removed, and the files containing the coordinates of PCI and thrombin were merged to form a complex using the Biopolymer module of Insight II. Keeping the P1 Arg fixed, PCI was fit manually into the active site cleft of thrombin by adjusting the torsion angle of the $C_\gamma-C_\beta$ bond. When obviously deleterious interactions between PCI and thrombin were alleviated, energy minimizations were begun. Steepest descent minimizations *in vacuo* were performed to a maximum derivative of 10 kcal/(mol·Å), with only the reactive site loop allowed to move. Next the backbone of thrombin was fixed, with the side chains allowed to move. Finally, the entire thrombin molecule and the portions of PCI in contact with thrombin were allowed to move. As thrombin and PCI moved relative to each other, some side chains between the molecules were trapped in constrained conformations which were relieved by manually adjusting torsion angles. The complex was surrounded by a 3 Å shell of water and minimized using Discover on the CRAY-YMP supercomputer at the North Carolina Supercomputing Center (Research Triangle Park, NC). Identical protocols were followed for the formation of APC−PCI and factor Xa−PCI complexes, using the PCI structure obtained from the thrombin−PCI complex as a starting point.

Simulated Mutagenesis of PCI. To estimate the local effects of mutagenesis of the reactive site loop in a complex, molecular modeling was again employed. The model of PCI was truncated to include only residues in contact with thrombin, and the ends were fixed so that the truncated portion of PCI could be superimposed back onto intact PCI. This greatly reduced the computation time required. The portions of PCI in contact with thrombin were still allowed to move as well as the reactive site loop and thrombin itself. Amino acid substitutions were made by using Biopolymer, and the mutated complex was minimized for 400 iterations of steepest descent *in vacuo*. The effect of mutation was determined by superimposing the wild-type complex on the mutated complex. Similar analysis was performed using the APC−PCI and factor Xa−PCI complexes.

RESULTS AND DISCUSSION

Molecular Model of PCI. The model of PCI was very similar to the model generated for HCII and to the crystal structure of AT with an rms of 3.54 between PCI and AT. A molecular model of PCI generated by Kuhn et al. (1990) did not have an intact reactive site loop and was not useful for comparison in this study. The reactive site loop of PCI has the same number of residues as AT and three more than HCII, resulting in a more extended structure than seen in the HCII model. The amino-terminal, end of the reactive site loop of PCI was partially inserted into the β-sheet of the body of PCI as is predicted for serpins (Carrell et al., 1994; Hopkins et al., 1993; Wei et al., 1994). In the crystal structure of AT, the P1 Arg residue points toward the body of AT in an orientation that would not be compatible with complex formation. In the absence of a good template for the reactive site loop, a functional approach was taken. We assumed that, to be active, the reactive site loop of PCI must have a conformation which would allow it to insert into the active site cleft of thrombin. Thrombin was chosen due to the availability of a crystal structure and due to the fact that the accessibility of its active site cleft is the most restrictive of the three proteases. The reactive site loop in our docked complex may not have the conformation present in native, uncomplexes PCI. However, we feel that it is a good approximation of the conformation the reactive site loop must assume in a complex with a protease.

Molecular Models of APC and Factor Xa. As expected, the final models of APC and factor Xa had high structural homology with thrombin. The rms variances between the backbone atoms of thrombin and homologous regions of APC and factor Xa were 1.13 and 1.10, respectively, consistent with the highly conserved nature of serine proteases. Our model had a rms variance of 2.55 with the model generated by Fisher et al. [pdb1pcu (Fisher et al., 1994)] with most of the differences occurring in surface loops of two molecules. The active site triad of each protease had the expected geometry and interatomic distances both before and after docking to PCI. APC has a region homologous to the autolysis loop (149A−149D) in thrombin but lacks a region similar to the 60-insertion loop of thrombin (60A−60I). Factor Xa lacks both loops and thus has a very exposed active site. Comparison of these three modeled structures suggests that thrombin may form the most stable complexes by having loops on both sides of the reactive site loop of PCI, thus grasping PCI in a "pincher-like" hold and possibly preventing water from reaching the tetrahedral bond formed between thrombin and PCI (Figure 1). This is supported by the fact that deleting residues in either the autolysis loop (146−148) or the 60-insertion loop (60B−60D) of thrombin results in 2 orders of magnitude less activity with AT (Le Bonniec et al., 1992, 1993). Furthermore, the rate of inhibition of the three proteases with PCI decreases with decreasing contacts between loops of the protease and the reactive site loop of PCI in the order thrombin ($k'/[I] = 3 \times 10^7$ M^- min^{-1}) > APC ($k'/[I] = 1 \times 10^6$ M^{-1} min^{-1}) > factor Xa ($k'/[I] = 2 \times 10^5$ M^{-1} min^{-1}) (Cooper & Church, 1995). A similar trend is seen with AT in which the rate of inhibition with thrombin ($k'/[I] = 1 \times 10^9$ M^{-1} min^{-1}) is greater than that with factor Xa ($k'/[I] = 2 \times 10^8$ M^{-1} min^{-1}) in the presence of heparin (Jordan et al., 1980; Pratt & Church, 1992).

Correlation between Structure and Activity in PCI−Protease Complexes. Examination of the protease residues within 5 Å of the P3−P3′ residues of PCI revealed that most of the interactions were between P3−P1 and the protease. There were fewer interactions between P1′−P3′ and the protease, consistent with the kinetic observation that mutations in these residues had minimal effect on PCI inhibitory activity. In each modeled complex the P1 Arg remained inserted into the S1 pocket and interacted with Asp_{189} of the protease. The P1 carbonyl carbon−Ser_{195} oxygen distances were 3.58, 3.15, and 4.16 Å for PCI complexed with thrombin, APC, and factor Xa, respectively. This is a reasonable distance considering that a covalent bond is not formed between protease and serpin in these models. In the PPACK-thrombin crystal structure this distance is 1.5 Å (Bode et al., 1992), the distance of a covalent bond. In trypsin complexes with Kunitz inhibitors values from 1.4 to 2.6 Å have been reported (Janin & Chothia, 1976; Sweet et

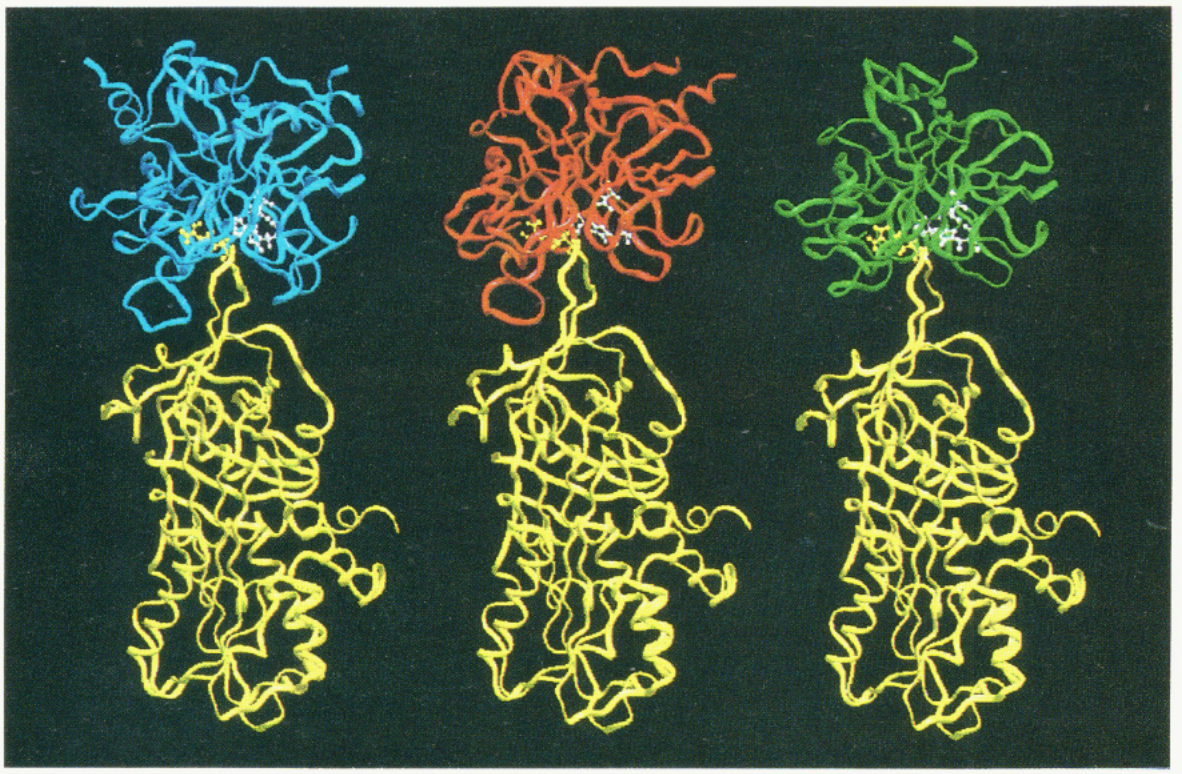

FIGURE 1: Molecular models of docked complexes between PCI and three serine proteases. PCI is in yellow in each complex. Thrombin is in blue (left), APC is in red (center), and factor Xa is in green (right). The P1 Arg of PCI is shown in yellow in each complex, and the active site triads of each protease are in white.

al., 1974). The planar P1−P1′ carbonyl bond should be perpendicular to the attacking active site Ser_{195}. The observed angles between the Ser_{195} oxygen−P1 carbonyl carbon−P1 α carbon were 82.9°, 92.9°, and 93.8° in complexes with thrombin, APC, and factor Xa, respectively.

Kinetically, the best inhibitors of APC and factor Xa, relative to wild-type rPCI, were the P3 reactive site mutants T352R (1.8 and 5.9, respectively) and T352R/R357D (1.2 and 4.0, respectively) (Figure 2). T352R/R357P was better than wild-type rPCI at inhibiting factor Xa (5.6) but was not any better with APC (0.7). Thr_{352} does not interact appreciably with residues in any of the proteases. However, when this residue is changed to Arg in the model, contacts with the conserved Glu_{217} of each protease are introduced (Figure 3). This would be consistent with the increase in activity observed in mutations containing T352R with both APC and factor Xa (Figure 2). Surprisingly, mutants with T352R were on average half as effective as wild-type rPCI in inhibiting thrombin. This observation held true with three different preparations of each mutant and four different batches of thrombin. At this time we do not have a direct explanation for the lack of increase in thrombin inhibition by P3 Arg-containing mutants. Glu_{217} forms a salt bridge with Lys_{224} in thrombin, which may repel a P3 Arg residue (Bode et al., 1992). Residue Arg_{173} of thrombin may also repel a P3 Arg in PCI, whereas APC and factor Xa have a less restrictive Ser at this position (Figure 3). Another possibility is that, given the constraints on the reactive site loop of PCI, a bulky group like Arg placed between two Phe residues is difficult to wedge between the autolysis and 60-insertion loops guarding the entrance to the active site of thrombin. This would be consistent with the observation that factor Xa showed the greatest increase with the P3 Arg mutants and has the most accessible active site. APC had a more moderate increase and has a less accessible active site, and thrombin showed no increase and has the most restricted active site.

It has been shown by mutagenesis of Glu_{192} to Gln that this residue is important in both thrombin and APC recognition of the P3 residue in peptides and physiological substrates (Guinto et al., 1994; Rezaie & Esmon, 1993). In our model a P3 Arg in PCI is capable of interacting with either residue Glu_{192} or Glu_{217} of thrombin or APC; however, we feel that interaction with Glu_{217} is more likely for the following reasons. Factor Xa, with Gln_{192}, is stimulated the most by Arg at the P3 residue, inconsistent with a P3−Gln_{192} interaction. In thrombin, Glu_{217} is in a salt bridge with Lys_{224} whereas Glu_{192} is unpaired. The converse is true in our APC model, with Glu_{217} unpaired and Glu_{192} forming a salt bridge with Arg_{147}. This salt bridge is not present in the model of APC generated by Fisher et al. (1994); however, this region of APC was the most disparate between the models, with our model looking more like that of thrombin.

Interaction of P3 with Glu_{217} is kinetically consistent with this residue being paired in thrombin and free in APC. Given the kinetic data, the fact that factor Xa has Gln at residue 192, and the molecular models, we feel that Glu_{217} is the most logical candidate to interact with the P3 residue of PCI. The discrepancies between the kinetic data using small peptides and the reactive site PCI mutants may be due to flexibility in the ends of the peptide, while corresponding residues in a serpin are restricted by the reactive site loop bending back into the body of the serpin. Furthermore, in both thrombin and APC the E192Q mutation results in a dramatic increase in its rate of inhibition with multiple serpins and Kunitz-type inhibitors; these data suggest a general mechanism independent of the P3 residue of the inhibitor (Guinto et al., 1994; Rezaie & Esmon, 1993).

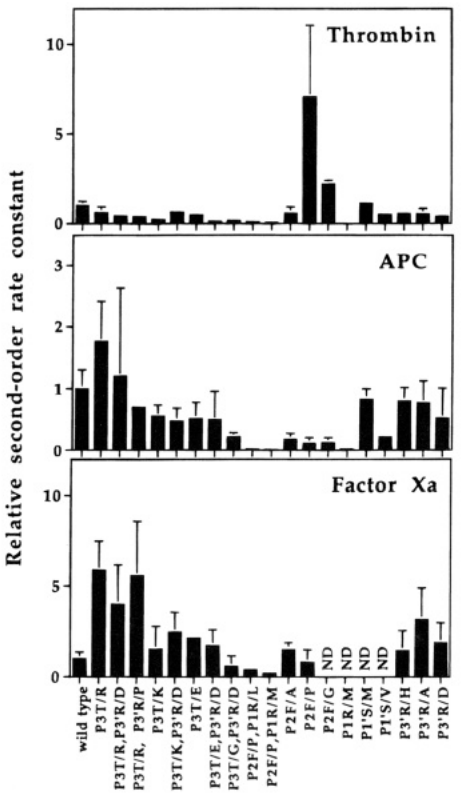

FIGURE 2: Relative activities of wild-type rPCI and reactive site loop mutants with thrombin, APC, and factor Xa. Assays were performed in the presence of optimum heparin concentrations, and activity values are expressed relative to wild-type rPCI. Each assay was performed two to four times in triplicate. Activities for the mutations P2F/G, P1R/M, P1'S/M, and P1'S/V were reported previously (Phillips et al., 1994). Activities for the mutations P2F/A and P2F/P and the double mutants P2F/P,P1R/L and P2F/P,P1R/M were also reported previously (Copper & Church, 1995). ND = not determined.

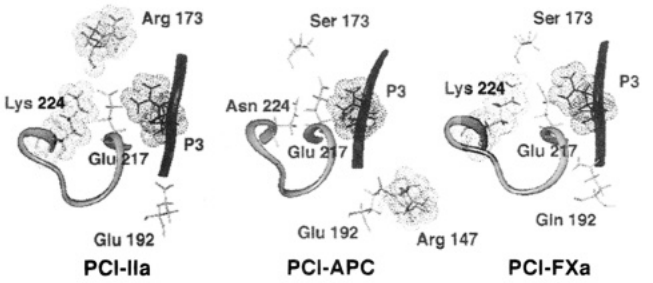

FIGURE 3: Interactions between the P3 residue of PCI with thrombin (left), APC (center), and factor Xa (right) in energy-minimized models of docked complexes. In each figure the P3 residue of PCI (black) was changed to Arg to mimic the effects of mutagenesis. Residues in contact with the P3 Arg in each complex are shown. Van der Waals radii of positively charged amino acids are displayed.

The P2 Phe of PCI is surrounded by a hydrophobic pocket consisting of the thrombin 60-insertion loop residues Tyr_{60A}, Trp_{60D}, and Lys_{60F} and the active site His_{57} (Figure 4). Changing the P2 Phe of PCI to Pro or Gly increases activity with thrombin 7- and 2-fold, respectively (Figure 2). Steric effects would appear to be the primary explanation for this observation; however, Ala is smaller than Phe and Pro, yet did not appreciably increase activity when inserted at the P2 position of PCI. This suggests that decreased steric hindrance and either flexibility (Gly) or a kinked backbone (Pro) at the P2 position contribute to increases in PCI inhibition of thrombin. Removing residues 60B−60D of

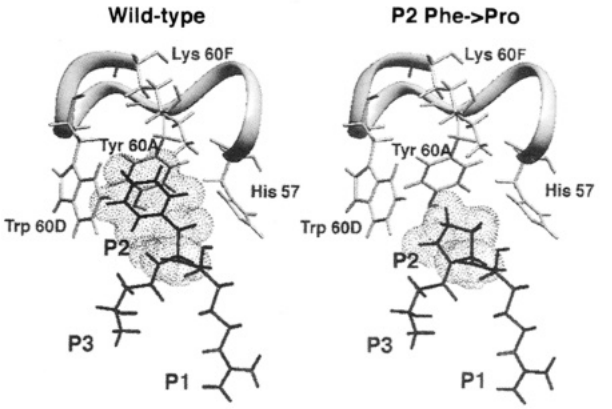

FIGURE 4: P2−thrombin interactions in a molecular model of a PCI−thrombin complex. The P1 through P3 residues of PCI (black residues) are on the bottom, and the 60-insertion loop of thrombin (gray ribbon) is on top in each figure. On the left is a complex between wild-type PCI and thrombin. On the right the P2 Phe of PCI has been replaced with Pro. The van der Waals radii of both P2 residues are indicated, and the reactive site loop of PCI is oriented parallel to the page.

thrombin resulted in a 100-fold decrease in AT inhibition (Le Bonniec et al., 1993), emphasizing the importance of these interactions in stabilizing serpin−protease complex formation. APC and factor Xa both lack the 60-insertion loop of thrombin, and instead the P2 Phe of PCI interacts weakly with His 57 and Thr 99 in APC and His 57 and Tyr 99 in factor Xa. Mutagenesis of the P2 Phe in PCI did not increase inhibitory activity with either APC or factor Xa (Figure 2). The absence of the 60-insertion loop of thrombin in these proteases may make it easier to accommodate a large aromatic Phe side chain in wild-type PCI.

Replacing the P3′ Arg of PCI with Asp on average decreased activity with thrombin and APC but had little effect on factor Xa inhibition. Introduction of negative charges at both P3 and P3′, T352E/R357D, generated the worst inhibitor of thrombin in this study, with a relative activity of 0.1 (Figure 2). This double mutant, with negatively charged P3 and P3′ residues, is similar to the thrombin activation site of protein C. These residues are repelled by Glu_{39} and Glu_{192} in thrombin, and this repulsion is thought to be decreased by thrombomodulin, stimulating thrombin cleavage of protein C (Le Bonniec & Esmon, 1991). The thrombin variant E39K has been shown to interact with the P3′ residue of peptide substrates (Le Bonniec et al., 1991). In APC and factor Xa the corresponding residues of thrombin's Glu_{39} are Lys and Glu, respectively. On the basis of mutagenesis results with thrombin we predicted that introduction of a negative charge at the P3′ of PCI would increase activity with APC and decrease activity with thrombin or factor Xa. Substitutions of Asp at P3′ did not have the predicted effect on PCI activity with either enzyme, suggesting that P3′ does not interact with residue 39 in any of these proteases. Consistent with these kinetic data, it appears that the P3′ Arg of PCI does not interact with Glu_{39} of thrombin but is directed toward the body of PCI (Figure 5). There is precedence for this in chymotrypsin inhibitor 2, in which the P3′ Arg residue points toward the body of the serpin and forms a salt bridge with the P1′ Glu (Jackson & Fersht, 1994). This salt bridge is thought to enhance the global stability of the reactive site loop as determined by increased guanidinium chloride induced denaturation of a mutant form of chymotrypsin inhibitor 2 with Glu at the P3′ position. In contrast with

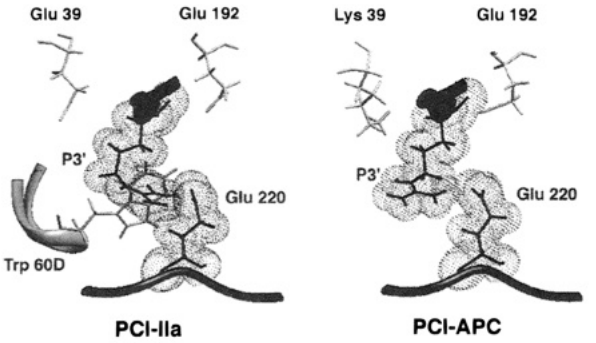

FIGURE 5: Potential salt bridge between the P3′ Arg and Glu_{220} of PCI (black) in molecular models of docked complexes with thrombin (left) and APC (right). Residues 192 and 39 of thrombin and APC are also shown for reference. The 60-insertion loop of thrombin also contacts the P3′ residue of PCI and is shown (gray ribbon).

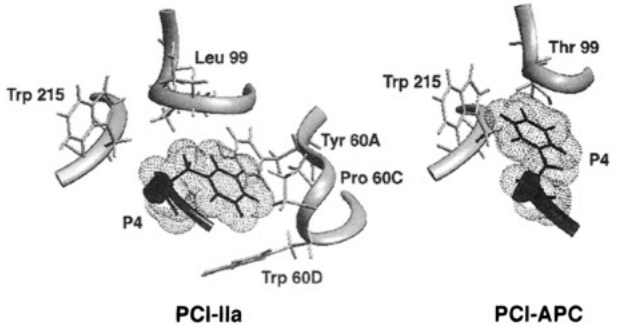

FIGURE 6: Molecular models demonstrating the interactions between the P4 residue of PCI with thrombin (left) and APC (right). The van der Waals radii of the P4 Phe of PCI are indicated in each model, and the reactive site loop is oriented perpendicular to the page (black ribbon). Thrombin and APC residues (gray) within 4 Å are displayed.

PCI, mutations at P3′ also resulted in dramatic decreases in inhibitory activity.

Several additional interactions between PCI and thrombin were predicted from the model that point to future possible mutagenesis experiments. Both the P4 and P4′ residues insert directly into the edges of the active site of thrombin. PCI's P4 Phe_{351} appears to interact with loop 97−100 of each protease. In thrombin this loop has an extra residue, forcing Phe_{351} into a different conformation which brings it into contact with Tyr_{60A} and Pro_{60C} of the 60-insertion loop of thrombin (Figure 6). Changing this residue to Ala is predicted to have the greatest effect on thrombin because of this increased steric hindrance; however, APC and factor Xa inhibition may also be increased with P4 Ala.

P4′ is a Leu in PCI and interacts with residues in the protease more than the P3′ Arg, including residue 39. In several serpins this residue is a Pro, and modeling this substitution shows decreased steric hindrance and a kinked backbone which may aid in bending the reactive site loop away from the active site of the protease. The P4′ residue in plasminogen activator inhibitor (PAI-1) is Glu and is important in the interactions between tissue plasminogen activator and PAI-1 (Madison et al., 1989, 1990).

The heparin-binding domain of PCI is located on the H-helix instead of the D-helix as seen in HCII and AT (Shirk et al., 1994). Changing Arg_{269} and Lys_{270} to Ala resulted in a 50% and 70% decrease in heparin-stimulated inhibition of APC and thrombin, respectively (Shirk et al., 1994). The last residue in the H-helix, Arg_{278}, is conserved in many

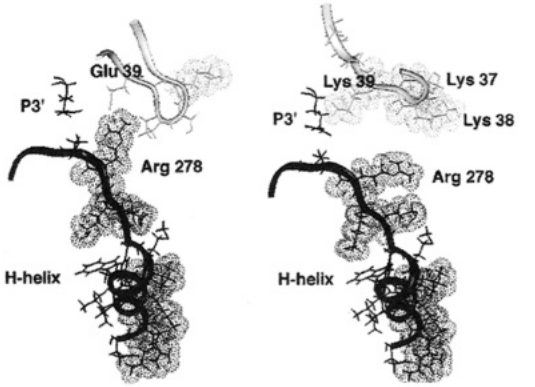

FIGURE 7: Molecular models of the H-helix of PCI (black ribbon) with thrombin (left figure, gray ribbon) and APC (right figure, gray ribbon). The van der Waals radii of positively charged amino acids are shown. The reactive site loop P3′ Arg of PCI is also shown for reference.

serpins with the exception of AT. This residue appears to form a salt bridge with Glu_{39} in thrombin (Figure 7). The corresponding region of APC consists of Lys_{37}, Lys_{38}, and Lys_{39}, which is a putative heparin-binding domain. In the PCI−APC complex, Arg_{278} appears to be repelled by this cluster of Lys residues (Figure 7). PCI is the only heparin-stimulated serpin which inhibits APC, and the proximity of the heparin-binding domains in the model suggests that heparin may act by bridging the H-helix of PCI and residues 37−39 of APC, neutralizing the positive charges between PCI and APC and thus accelerating the PCI−APC reaction. Changing Arg_{278} of PCI, or Lys_{39} of APC, to a Glu should stimulate the heparin-independent APC−PCI reaction if this model is correct. This would also be one of the first contacts between a serpin and protease identified outside of the reactive site loop of a serpin.

Combining molecular modeling and site-directed mutagenesis generates a powerful tool in understanding protein−protein interactions. As PCI can form complexes with several serine proteases, more information can be gleaned from each mutation. Our molecular models are consistent with the kinetic data obtained from the PCI reactive site mutants and other biochemical observations. We feel that these models will prove useful in predicting other points of contact between serpins and proteases which may not have been noted otherwise.

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