Role of the P_2 Residue in Determining the Specificity of Serpins†

Marylyn Z. Djie, Bernard F. Le Bonniec, Paul C. R. Hopkins, Karsten Hipler, and Stuart R. Stone*

Department of Haematology, Cambridge University, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

Received November 14, 1995; Revised Manuscript Received May 15, 1996⊗

ABSTRACT: The importance of the P_2 residue in determining serpin specificity was examined by making a series of substitutions in the P_2 position of recombinant α_1-antichymotrypsin that contained an arginine P_1 residue. The importance of the P_2 residue in governing the association rate constant (k_{on}) of the serpin varied with the protease examined. For trypsin, the P_2 residue played a relatively minor role, whereas the nature of this residue markedly influenced the rates of inhibition of thrombin, factor Xa, and APC. A 1000-fold difference in k_{on} values was observed between the fastest (P_2 proline) and the slowest (P_2 threonine) inhibitors of thrombin. Similar differences were observed with factor Xa; the best inhibitor (P_2 glycine) displayed a 200-fold higher k_{on} value than the poorest (P_2 threonine). The nature of the P_2 residue also affected whether the interaction of the serpin with the protease resulted in inhibition of the protease or cleavage of the serpin; a P_2 proline residue increased the rate of cleavage of α_1-antichymotrypsin by trypsin. By using mutants of thrombin, it was possible to show that the B-insertion loop, which partially occludes the active site, is important in determining the P_2 specificity of this enzyme. Deletion of three amino acids from this loop yielded a protease (des-PPW) that became more like trypsin in its specificity. In addition, it was shown that Glu^{192} dramatically restricts thrombin's ability to accommodate a threonine in the P_2 position. Taken together, the results demonstrated the importance of complementary interactions between the P_2 residue of the serpin and the S_2 binding site of the protease in regulating the specific interaction between serpin and protease.

Serpins are a family of serine proteases inhibitors that share a common tertiary structure in which eight α helices and three β sheets combine to form an ellipsoid molecule. Although members of this superfamily of proteins are structurally similar, they have a variety of roles in the control of diverse systems, such as blood coagulation, fibrinolysis, complement activation, and inflammation (Travis & Salvesen, 1983; Huber & Carrell, 1989; Potempa et al., 1994). While the elements of secondary structure have a high degree of sequence identity, the reactive site loop (RSL)[1] is a region whose sequence is hypervariable. This region of the serpin binds to the active site of its target protease and, thus, its sequence determines the specificity of the inhibitor. The P_1 residue[2] is known to be a major determinant of serpin specificity. For instance, replacement of the P_1 methionine of α_1-antitrypsin by arginine in the Pittsburgh mutant results in an efficient inhibitor of thrombin and can lead to a fatal hemorrhagic disorder (Owen et al., 1983). Natural P_1 variants of antithrombin (Owen et al., 1991) and C1 inhibitor (Skriver et al., 1989) have also illustrated the importance of this residue in the function of these serpins. Protein engineering studies have confirmed the important role of the P_1 residue in regulating serpin specificity (Travis et al., 1985; York et al., 1991; Derechin et al., 1990; Eldering et al., 1992; Sherman et al., 1992). Although these studies with both natural and recombinant mutants illustrate the significant contribution of the P_1 residue in the formation of serpin−protease complexes, this residue is not the sole determinant of specificity and its importance varies with the serpin−protease combination. For example, trypsin has a marked preference for basic P_1 residues in substrates, but it is efficiently inhibited by α_1-antitrypsin which has methionine in the P_1 position. Although α_1-antitrypsin and α_1-antichymotrypsin (ACT) share over 45% sequence identity, replacement of the P_1 leucine residue of ACT by a methionine (as in α_1-antitrypsin) does not lead to an ACT variant that is able inhibit the target of α_1-antitrypsin (neutrophil elastase) efficiently (Rubin et al., 1990). These results suggest that for some serpin−protease interactions, other residues play a considerable role in determining specificity.

A number of studies suggest that the P_2 residue may be among those playing such a role. Schapira et al. (1987) found that mutation of the P_2 proline of Pittsburgh α_1-antitrypsin (P_1-Arg-α_1-antitrypsin) to alanine increased its rate of reaction with kallikrein relative to that observed with thrombin. York et al. (1991) demonstrated that the relative rate of inhibition of urokinase plasminogen activator and tissue plasminogen activator by plasminogen activator inhibitor 1 could be markedly influenced by the nature of the P_2 residue. Sheffield and Blajchman (1994) found that large hydrophobic and charged P_2 residues in antithrombin disrupted complex formation with thrombin. Exchanging the P_2 phenylalanine of protein C inhibitor for a glycine improved the ability of this protein to inhibit thrombin and urokinase but led to a lower activity toward activated protein

† This work was supported by grants from the MRC and British Heart Foundation.

* Address correspondence to this author.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1996.

[1] Abbreviations: ACT, α_1-antichymotrypsin; APC, activated protein C; RSL, reactive-site loop; SI, stoichiometry of inhibition; P_1-Arg-α_1-antitrypsin, α_1-antitrypsin in which the P_1 methionine residue has been replaced by an arginine; E192Q, thrombin in which Glu^{192} (chymotrypsin numbering system; Bode et al., 1989) has been replaced by a glutamine; des-PPW, a mutant thrombin in which the three residues (Pro^{60B}, Pro^{60C}, and Trp^{60D}) have been deleted.

[2] The nomenclature for residues within the reactive site loop is based on that outlined by Schechter and Berger (1967) for the substrates of proteases. The residues are numbered from the cleaved bond (P_1-P_1') as follows: P_n-...-P_3-P_2-P_1-P_1'-P_2'-P_3'-...-P_n'.

S0006-2960(95)02717-6 CCC: $12.00

C (APC) and trypsin (Phillips et al., 1994). In the present study, the contribution of the P_2 residue to serpin specificity has been further investigated. For these investigations, we have utilized recombinant ACT. Although replacement of the P_1 leucine of recombinant ACT by arginine led to a serpin that was able to inhibit thrombin, the association rate constant (k_{on}) with thrombin was lower than those observed for antithrombin, protease nexin 1, or P_1-Arg-α_1-antitrypsin (Rubin et al., 1990), suggesting that other residues may be important for the inhibition of thrombin. To investigate additional determinants responsible for specificity, double mutants were constructed in which the P_1 leucine was replaced by arginine and the P_2 leucine was replaced by various amino acids. The inhibitory characteristics of these P_2 mutants with a number of serine proteases (thrombin, factor Xa, APC, and trypsin) were determined. The importance of interactions with the P_2 residue was also investigated by utilizing two mutants of thrombin which have an altered specificity for P_2 residues (E192Q and des-PPW; Le Bonniec & Esmon, 1991; Le Bonniec et al., 1993). The results obtained illustrated that interactions with the P_2 residue do indeed make a major contribution to serpin specificity. The influence of the P_2 residue with thrombin was particularly striking; a 1000-fold difference in k_{on} values between the best (proline) and worst (threonine) P_2 residues was observed.

EXPERIMENTAL PROCEDURES

Materials. Fast-flow Q-Sepharose and fibrous cellulose powder CF11 were obtained from Pharmacia (Milton Keynes, U.K.) and Whatman Laboratories (Maidstone, U.K.), respectively. The chromogenic substrates *N*-benzoyl-Ile-Glu-(γ-OR)-Gly-Arg-*p*-nitroanilide where R = H (50%) and CH_3 (50%) (S-2222), D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238), D-Val-Leu-Arg-*p*-nitroanilide (S-2266), D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), and D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) were obtained from Chromogenix (Mölndahl, Sweden). Human α-thrombin, the thrombin mutants E192Q and des-PPW, as well as bovine factor Xa, were prepared as described previously (Stone & Hofsteenge, 1986; Le Bonniec & Esmon, 1991; Le Bonniec et al., 1992, 1993). Human activated protein C (APC) was a gift from Drs. J. Stenflo and A. Öhlin (University of Lund, Malmö, Sweden). Bovine pancreatic trypsin (TPCK-treated) and the venom from *Bitis arietans* were purchased from Worthington Enzymes (Twyford, U.K.) and Sigma (Poole, U.K.), respectively. The concentrations of thrombin and trypsin were determined by active-site titration with *p*-nitrophenyl *p*′-guanidinobenzoate (Chase & Shaw, 1970). The cDNA for wild-type ACT and the plasmid used for the expression of the mutant ACT-LR[3] (Rubin et al., 1990) were gifts from Dr. H. Rubin (University of Pennsylvania, Philadelphia, PA). Oligonucleotides were purchased from the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge.

Expression of ACT Variants. ACT-LR was expressed as described by Rubin et al. (1990) using the plasmid pZMS ACT. Other mutants were expressed using a vector derived from pJLA502 (Medac, Hamburg, Germany). This vector contains the temperature-sensitive cI^{ts857} repressor gene and the λP_L and P_R promoters in tandem preceding the cloning site, which is followed by the fd terminator. The cDNA encoding ACT was inserted into this vector in a two-step procedure. Initially, an *Xba*I−*Bgl*II fragment from pZMS ACT encoding the N-terminal region of ACT was ligated into pJLA502 using an *Xba*I site that had previously been created in the ribosome binding sequence of pJLA502. This vector was cut using *Bst*XI (in the coding sequence) and *Eco*RI (after the end of the ACT fragment). A *Bst*XI−*Eco*RI fragment encoding the C-terminal region of ACT was then obtained from the replicative form of M13 phage containing ACT cDNA and cloned into the cut expression vector. Site-directed mutagenesis was performed using the method of Kunkel (1985). The presence of the desired mutation and the absence of others were confirmed by sequencing of the expression vector.

Purification of ACT Variants. Recombinant ACT was expressed and purified according to the method of Rubin et al. (1990) with slight modifications. The cells from six 1-L cultures were harvested by centrifugation, resuspended in 40 mL of 10 mM potassium phosphate buffer, pH 7.5, containing 50 mM KCl, 1 mM phenylmethanesulfonyl chloride, 1 mM EDTA, and 1 mM β-mercaptoethanol (buffer A) and immediately stored at −70 °C. The frozen cells were allowed to defrost and then disrupted using a French press. Cell debris was removed by centrifugation and the supernatant was loaded onto a Q-Sepharose column (2.5 × 25 cm) previously equilibrated with buffer A. The column was washed with 5 column volumes of buffer A and the protein was eluted with a KCl gradient (0.05−0.5 M; 1600 mL). The fractions containing recombinant ACT were identified by rocket immunoelectrophoresis, pooled, and diluted using buffer A, pH 6.8, to an ionic strength of about 50 mM. This sample was then loaded onto a 2.5- × 15-cm column of DNA−cellulose (Alberts & Herrick, 1971) which had been equilibrated with buffer A, pH 6.8. The column was washed with 5 column volumes of equilibration buffer before ACT was eluted from the column with 0.4 M KCl; fractions containing ACT were identified using rocket immunoelectrophoresis and pooled. The pooled fractions were diluted using buffer A, pH 7.5, until the KCl concentration was approximately 50 mM. This solution was loaded onto a Q-Sepharose column (1.25 × 10 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl. The column was washed with 5 column volumes of this buffer and the ACT was eluted with 50 mM Tris-HCl buffer, pH 7.5, containing 0.4 M NaCl. Recombinant ACT eluted as a single absorbance peak (280 nm). SDS−PAGE on 10−20% polyacrylamide gels (Laemmli, 1970) indicated that each of the preparations was essentially pure. The concentration of ACT was estimated from its absorbance at 280 nm using the extinction coefficient ($\epsilon^{1\%}$) of 6.2 cm^{-1} (Travis & Morii, 1981).

Analysis of Complex Formation by SDS−PAGE. The ability of the ACT variants to form SDS-stable complexes with thrombin and trypsin was assessed by incubating the variants (2 μM) with either thrombin (1 μM) or trypsin (1 μM) in 50 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl and 0.2% (w/v) poly(ethylene glycol) (M_r 6000) for 30 min at room temperature. This incubation mixture was then analyzed by SDS−PAGE on 10−20% gels (Laemmli, 1970). ACT cleaved within its reactive-site loop was generated by incubating wild-type ACT (200 μg) with the venom from *B. arietans* (0.6 μg) for 2 h at 37 °C in 30 mM

[3] Variants of ACT are identified by using the single-letter code for the sequence of their P_2 and P_1 residues; for example, ACT-LR and ACT-PR contain the P_2-P_1 sequences Leu-Arg and Pro-Arg, respectively. All other residues were constant.

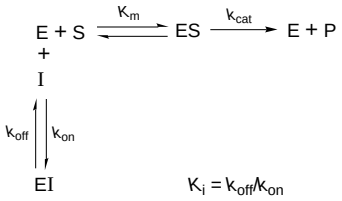
potassium phosphate buffer, pH 7.0, containing 30 mM NaCl and 5 mM EDTA. N-Terminal sequence analysis indicated that this treatment led to cleavage of ACT between the P_2' alanine and P_3' leucine (D. A. Lomas, personal communication). This RSL-cleaved material was used as a standard along with uncleaved ACT.

Amidolytic Assays. All assays were performed at 37 °C in 50 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl, 0.2% (w/v) poly(ethylene glycol) (M_r 6000), and 0.1% (w/v) bovine serum albumin (kinetics buffer). Assays for activated protein C (APC) contained 5 mM $CaCl_2$ in kinetics buffer. The assays also contained the following peptidyl *p*-nitroanilide substrates: S-2238 or S-2266 for thrombin variants, S-2288 or S-2302 for trypsin, S-2222 for factor Xa, and S-2238 or S-2266 for APC. The production of *p*-nitroaniline due to the cleavage of the peptidyl *p*-nitroanilide substrates was monitored by measuring the increase in absorbance at 405 nm using a Molecular Devices microplate reader for serpin titrations or between 400 and 410 nm using a Hewlett-Packard 8452A spectrophotometer for progress-curve kinetics.

Determination of the Stoichiometry of Inhibition. SI values of the ACT variants were determined as described previously (Hermans & Stone, 1993). Different concentrations of serpins were incubated with about 0.1 μM thrombin or trypsin in kinetics buffer at 37 °C for a period of time sufficient to ensure that complex formation was complete (3 −5 h). The residual amidolytic activity was then determined by the addition of 200 μM S-2266 (thrombin) or S-2302 (trypsin). Linear regression analysis of the variation of protease activity with the concentration of serpin yielded the estimates for the stoichiometry of inhibition (numbers of mole of serpin required to inhibit 1 mol of protease).

Slow-Binding Inhibition Assays. A slow-binding inhibition experiment consisted of seven assays: one in the absence of serpin and six different concentrations chosen such that significant inhibition would be observed over the time of the experiment (3 h). The assays were initiated by the addition of enzyme; the concentration of enzyme was adjusted such that initial velocity in the absence of inhibitor was approximately 0.2 μM min^{-1}. The substrate used and its concentration (200−400 μM) were varied according to the apparent half-life of the complex formation. As outlined below, the rate of complex formation in the presence of substrate will be inversely proportional to $(1 + [S]/K_m)$; thus, substrates with higher K_m values were used with serpins with lower association rate constants so as to minimize the competing effect of the substrate.

Scheme 1



Analysis of Slow-Binding Inhibition Data. The inhibition of proteases by ACT variants was analyzed in terms of Scheme 1, where E, I, S, and P represent thrombin, serpin, substrate, and substrate cleavage products, respectively. For this mechanism, the progress curve of formation of product will be described by the following equation (Morrison, 1982):

$$P = v_s t + \frac{v_i - v_s}{k'}[1 - \exp(-k't)] \quad (1)$$

where P is the concentration of product at time t, k' is an apparent first-order rate constant, and v_i and v_s are the initial and steady-state velocities, respectively. When the concentration of inhibitor is much greater than its apparent overall inhibition constant (K_i'), v_s will be negligible and eq 1 simplifies to

$$P = \frac{v_i}{k'}[1 - \exp(-k't)] \quad (2)$$

For the mechanism presented in Scheme 1, the dependence of v_s and k' on the inhibitor concentration and K_i' will be described by the following equations (Cha, 1975; Morrison & Walsh, 1988):

$$v_s = \frac{v_i}{1 + [I]/K_i'} \quad (3)$$

where $K_i = k_{off}/k_{on}$ and $K_i' = K_i(1 + [S]/K_m)$:

$$k' = \frac{k_{on}[I]}{1 + [S]/K_m} + k_{off} \quad (4)$$

The kinetic parameters that describe the inhibition caused by a serpin are its association rate constant (k_{on}) and K_i, the overall inhibition constant derived from the amount of inhibited enzyme at infinite time. Equations 3 and 4 were substituted into eq 1 and the data from a slow-binding inhibition experiment consisting of seven progress curves were fitted to this overall equation by nonlinear regression (Stone & Hofsteenge, 1986). Data for which the substrate utilization was greater than 10% were excluded from the regression analysis. From this analysis, estimates of K_i and k_{on} were obtained. It should be noted that the values used for [I] were the total concentration of serpin. The K_m values required for the calculations were determined using standard initial velocity techniques. The K_m values of S-2288 and S-2302 with trypsin were 19 and 134 μM, respectively. With thrombin, K_m values of 3.6 and 486 μM were obtained for S-2238 and S-2266, respectively, while the values for these two substrates with E192Q were, respectively, 18 and 522 μM. The substrate S-2238 exhibited a K_m value of 78 μM with des-PPW. The K_m of S-2222 with factor Xa was 547 μM. APC displayed K_m values of 315 and 222 μM with S-2238 and S-2266, respectively.

It was not possible to obtain an accurate estimate for K_i when the rate of formation of the inhibited complex was very slow. The K_i can only be accurately determined when the steady-state velocity (v_s) has been achieved during the time of the experiment with serpin concentrations not vastly greater than K_i'. In practice, this meant that it was in general only possible to determine K_i values with trypsin. For protease−serpin interactions with low k_{on} values ($< 10^3$ M^{-1} s^{-1}), an accurate value of K_i could not be determined and the estimate of k_{on} was also often unreliable. The reliability of the estimate of the k_{on} value could be improved by setting the value of v_s to 0 (eq 2). For these analyses, eq 4 with k_{-1} omitted was substituted into eq 2 and the data were analyzed by nonlinear regression as described above. Equa-

Table 1: Stoichiometry of Inhibition for P_2 Variants of ACT with Thrombin and Trypsin[a]

variant	SI values	
	thrombin	trypsin
ACT-LR	1.8	3.0
ACT-PL	b	48
ACT-AR	3.3	1.3
ACT-PR	2.9	106
ACT-VR	2.5	1.4
ACT-GR	2.3	1.9
ACT-TR	b	1.4
ACT-FR	4.0	1.1

[a] Values for the stoichiometry of inhibition (SI) were determined from titrations of the variants against thrombin and trypsin as described in Experimental Procedures. [b] The slow association rate constant of these variants with thrombin precluded the determination of their SI values.

tion 2 will describe the inhibition when the concentration of inhibitor is much greater than its K_i value; the concentrations of serpin used under these conditions (1−10 μM) were much greater than the average K_i values observed (< 10 nM).

Each slow-binding inhibition experiment was performed at least twice and the k_{on} values of the parameters given represent the weighted means of these determinations. The standard errors of values are reported as a percent of the estimated value and were obtained from the variance−covariance matrix of the regression analysis. Because of the large number of points used in progress-curve kinetics (about 30 points/curve were analyzed), the standard errors are underestimated (Duggleby & Morrison, 1978).

Molecular modeling was performed using the QUANTA software package (Molecular Simulations, Waltham, MA) together with the coordinates for trypsin (Marquart et al., 1983) and thrombin (Bode et al., 1989; Stubbs et al., 1992).

RESULTS

Stoichiometries for the Inhibition of Thrombin and Trypsin by ACT P_2 Variants. Each of the P_2 variants of ACT was titrated against thrombin and trypsin; the values for the stoichiometry of inhibition derived from these titrations are given in Table 1. Accurate determination of the stoichiometry of inhibition (SI) depends on the reaction between the inhibitor and enzyme being complete within the time of incubation of the serpin and proteases. The concentrations of thrombin and trypsin used for the titration experiments were 0.1 μM. With this concentration, the half-life for the formation of the stable complex will be about 2 h when the association rate constant (k_{on}) is 10^3 M^{-1} s^{-1}. Consequently, it was not possible to determine SI values when k_{on} values were less than 10^3 M^{-1} s^{-1} with the incubation times used (3−5 h), and SI values for ACT-PL and ACT-TR with thrombin could not be obtained. Although most variants displayed SI values with trypsin that were not much greater than 1, two variants (ACT-PL and ACT-PR) exhibited very large SI values (≥ 50) with trypsin (Table 1). The SI values of the P_2 variants with thrombin varied from 2 to 4 and were in general somewhat higher than the corresponding values with trypsin; ACT-PR, for which the SI value was considerably lower with thrombin than with trypsin, was the exception.

Analysis of Complex Formation by SDS−PAGE. The capacity of the ACT variants to form SDS-stable complexes with thrombin and trypsin was examined by incubating 2 μM mutant ACT with 1 μM enzyme for 30 min at room temperature, followed by SDS−PAGE analysis in 10−20% gels under reducing conditions. Complex formation was detected following incubation of thrombin with all ACT variants having a P_1 argininine. A major band with an apparent molecular mass equal to that of thrombin plus recombinant ACT (~70 kDa) was detected for all mutants except ACT-PL (Figure 1A). The absence of a prominent band corresponding to the complex with this mutant was presumably due to the slow rate of reaction of this variant with thrombin (see Table 2).

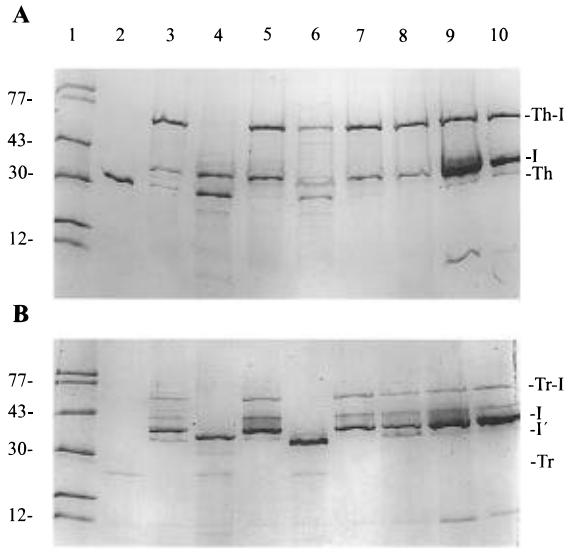


FIGURE 1: SDS−PAGE analysis of reactions of ACT P2 variants with thrombin and trypsin. (A) Thrombin. ACT P_2 variants (2 μM) were incubated with thrombin (1 μM) at room temperature for 30 min and the reaction products were analyzed by SDS−PAGE on 10−20% gels. Lane 1, molecular mass markers (apparent molecular masses in kilodaltons are indicated); lane 2, thrombin (1 μM); lanes 3−10, thrombin incubated with ACT-LR, ACT-PL, ACT-AR, ACT-PR, ACT-VR, ACT-GR, ACT-TR, and ACT-FR, respectively. The labels Th−I, I, and Th show the positions of migration the thrombin−serpin complex, uncleaved serpin, and thrombin, respectively. (B) Trypsin. ACT P_2 variants (2 μM) were incubated with trypsin (1 μM) at room temperature for 30 min and the reaction products were analyzed by SDS−PAGE on 10−20% gels. Lane 1, molecular mass markers (apparent molecular masses in kilodaltons are indicated); lane 2, trypsin (1 μM); lanes 3−10, trypsin incubated with ACT-LR, ACT-PL, ACT-AR, ACT-PR, ACT-VR, ACT-GR, ACT-TR, and ACT-FR, respectively. The labels Tr−I, I, I′, and Tr show the positions of migration of the trypsin−serpin complex, uncleaved serpin, serpin cleaved in the RSL, and trypsin, respectively.

ACT-PL and ACT-PR did not form SDS-stable complexes with trypsin to any appreciable extent but were cleaved. These two mutants, which possess a proline in the P_2 position, migrated with an apparent molecular mass corresponding to that of ACT cleaved within the RSL, while all other mutants were able to form complexes (Figure 1B). These results are in agreement with those obtained in the titration experiments, which indicated that these two mutants had SI values much greater than 1.

Kinetic Parameters for ACT P_2 Variants. Kinetic constants for the various ACT mutants with the different proteases with a preference for a P_1 arginine were determined using progress-curve kinetics. Analysis of the data yielded association rate constants (k_{on}) for most interactions (Table 2). In a number of cases, the formation of the inhibited complex occurred too slowly to permit an accurate estimation of k_{on} and only a limiting value for this constant could be

Table 2: Association Rate Constants for ACT P_2 Variants with Different Arginine-Specific Proteases[a]

	k_{on} (M^{-1} s^{-1})					
variant	factor Xa	trypsin	APC	thrombin	E192Q	des-PPW
ACT-PL	< 5.0	7.4×10^2 (7%)	< 5.0	1.3×10^1 (12%)	9.7×10^1 (16%)	< 5.0
ACT-LR	2.1×10^1 (4%)	4.1×10^5 (1%)	1.9×10^2 (2%)	1.8×10^3 (2%)	1.2×10^4 (4%)	8.0×10^1 (6%)
ACT-AR	6.0×10^2 (1%)	4.2×10^5 (2%)	7.8×10^1 (3%)	6.3×10^2 (1%)	5.1×10^3 (4%)	1.3×10^2 (2%)
ACT-PR	6.8×10^2 (2%)	4.4×10^5 (6%)	2.0×10^2 (6%)	3.3×10^4 (1%)	6.1×10^4 (2%)	3.8×10^2 (3%)
ACT-VR	1.8×10^1 (8%)	3.8×10^5 (7%)	3.8×10^2 (3%)	1.2×10^3 (2%)	1.1×10^4 (3%)	1.4×10^2 (2%)
ACT-GR	3.0×10^3 (1%)	5.5×10^5 (2%)	7.7×10^0 (6%)	1.5×10^3 (2%)	1.7×10^3 (2%)	1.2×10^2 (2%)
ACT-TR	1.5×10^1 (3%)	3.1×10^5 (2%)	1.1×10^2 (11%)	3.4×10^1 (2%)	1.8×10^3 (1%)	2.5×10^1 (4%)
ACT-FR	7.4×10^1 (3%)	3.1×10^5 (2%)	1.9×10^2 (8%)	2.2×10^2 (2%)	1.9×10^3 (2%)	2.8×10^1 (7%)

[a] Assays were performed as described in Experimental Procedures. Analysis of the data by nonlinear regression according to the equations for slow-binding inhibition yielded the estimates of the association rate constants (k_{on}) given in the table. The values given in parentheses after the values for k_{on} are the standard errors of the estimates obtained from the regression analysis expressed as a percentage of the estimate.

estimated. For the interactions with trypsin, it was also possible to determine K_i values for the P_2 variants with P_1 arginine residues. With the exception of ACT-PR, which exhibited a K_i value of 7.6 ± 0.1 nM, the K_i values obtained were all less than 1 nM (the weighted mean of six values was 0.44 ± 0.01 nM). For the interactions with other proteases, it was not possible to obtain accurate estimates for K_i.

Inhibition of Thrombin, Factor Xa, APC, and Trypsin by ACT P_2 Variants. The highest k_{on} values were obtained with trypsin. In addition, the rate of inhibition of trypsin was hardly affected by the nature of the P_2 residue. The mean value of k_{on} for seven mutants with trypsin was 4.0×10^5 M^{-1} s^{-1} and the k_{on} values of the slowest and fastest mutants differed from the mean by less than 40% (Table 2). In contrast, the nature of the P_2 residue had a profound effect on the rate of inhibition of thrombin; a 970-fold difference in the k_{on} values of the slowest and fastest variants was observed. The most favorable amino acid was proline; ACT-PR had a k_{on} value of 3.3×10^4 M^{-1} s^{-1} with thrombin. The worst P_2 residue was threonine; ACT-TR exhibited a k_{on} value of only 34 M^{-1} s^{-1} (Table 2).

For factor Xa inhibition, the largest k_{on} value was observed with the ACT-GR mutant (3.0×10^3 M^{-1} s^{-1}) and a difference of 170-fold was observed between the k_{on} for this variant and that for the slowest one (ACT-VR; $k_{on} = 18$ M^{-1} s^{-1}). In contrast to thrombin, factor Xa did not readily accommodate valine and leucine in the P_2 position; ACT-VR and ACT-LR had the lowest k_{on} values with factor Xa, whereas they reacted reasonably rapidly with thrombin (Table 2).

The association rate constants for the P_2 variants with APC were consistently lower than those observed with the other proteases. With the exception of the ACT-GR mutant, all the k_{on} values were approximately 10^2 M^{-1} s^{-1}, with the highest value of 3.8×10^2 M^{-1} s^{-1} being observed with a valine in P_2. The k_{on} value for the variant with a P_2 glycine (7.7 M^{-1} s^{-1}) was 50-fold lower (Table 2). The greatest selectivity for thrombin over APC was obtained with a glycine or proline in the P_2 position; the k_{on} values for both the ACT-GR and ACT-PR variants were 100-fold higher with thrombin. In contrast to thrombin, APC accommodated both threonine and phenylalanine in preference to glycine in the P_2 position; the k_{on} values for ACT-TR and ACT-FR were about 20-fold higher than that for ACT-GR with APC. An opposite preference was observed with thrombin; ACT-TR and ACT-FR exhibited the lowest k_{on} values with thrombin (Table 2).

Inhibition of Thrombin Mutants with Altered P_2 Specificities by ACT Variants. In order to investigate further the interactions of the P_2 residue of ACT with thrombin, the inhibition of two mutants of thrombin with altered P_2 specificities was examined. The mutants examined were (1) E192Q, in which the Glu^{192} is replaced by a glutamine; this residue is located at the entrance of the primary specificity pocket and the E192Q mutation affects the ability of thrombin to accommodate glycine and valine in the P_2 position (Le Bonniec & Esmon, 1991; Le Bonniec et al., 1992) and (2) des-PPW, in which the three residues Pro^{60B}, Pro^{60C}, and Trp^{60D} have been deleted; these residues comprise part of an enlarged loop, referred to as the 60-loop or B insertion, and the des-PPW deletion affects the preference of thrombin for proline in the P_2 position (Le Bonniec et al., 1993).

The k_{on} values of all P_2 variants with the E192Q mutant were higher than those observed with thrombin (Table 2). However, the magnitude of the difference between E192Q and thrombin varied. Whereas ACT-GR was only marginally faster with E192Q, the k_{on} value for ACT-TR variant was 53-fold higher with E192Q than with thrombin. The E192Q mutation reduced the P_2 specificity of thrombin. The difference in k_{on} values between the fastest (ACT-PR) and slowest (ACT-GR) was 36-fold compared with the 1000-fold difference observed between the slowest and fastest with thrombin (Table 2).

Compared to wild-type thrombin, the deletion mutant des-PPW exhibited k_{on} values with the ACT variants that were in general about 10-fold lower than those observed with thrombin. In addition, des-PPW had lost some of the specificity of thrombin; it appears to be more trypsin-like. Only small differences were observed in the k_{on} values for the variants having leucine, alanine, proline, valine, or glycine in the P_2 position (Table 2). However, des-PPW still retained, albeit to a smaller extent, the aversion of wild-type thrombin to a P_2 threonine or phenylalanine. The k_{on} values for ACT-TR and ACT-FR with des-PPW were about 15-fold lower than that of the most rapid inhibitor (ACT-PR).

The E192Q mutation in thrombin also alters the P_1 specificity of the protease. Although wild-type thrombin is only poorly inhibited by α_1-antitrypsin, which has a P_1 methionine and a P_2 proline, this serpin is an efficient inhibitor of E192Q; the k_{on} value for α_1-antitrypsin with E192Q is more than 1000-fold higher than that with wild-type thrombin (Le Bonniec et al., 1995). The mutant ACT-PL was constructed to test the hypothesis that interactions between a P_2 proline and glutamine at position 192 of thrombin allow a neutral residue to be accommodated in the P_1 position. A slow but detectable rate of inhibition of thrombin by ACT-PL (13 M^{-1} s^{-1}) was observed in spite of

the P_1 leucine residue, and the k_{on} value was 7-fold higher with the E192Q mutant. The difference in the k_{on} values of ACT-PL with thrombin and E192Q was much less than the 1000-fold difference in the k_{on} values observed with α_1-antitrypsin. Thus, if interactions between the P_2 proline and Gln^{192} enable E192Q to bind a P_1 methionine efficiently, such interactions are not sufficient to facilitate efficient binding of a P_1 leucine. The mutant ACT-PL failed to inhibit all other proteases tested except trypsin, for which a k_{on} value of 7.4×10^2 M^{-1} s^{-1} was obtained.

DISCUSSION

The P_2 Residue as a Determinant of Serpin Specificity. Although the importance of the P_1 residue in determining the specificity of serpins has been firmly established (Travis et al., 1985; Skriver et al., 1989; Derechin et al., 1990; Owen et al., 1991; Eldering et al., 1992; Sherman et al., 1992), the influence of the P_2 residue has been less extensively studied (Phillips et al., 1994; Sheffield & Blajchman, 1994; York et al., 1991). The results obtained in the present study illustrate that the P_2 residue can play a decisive role in serpin specificity. However, the importance of the P_2 residue varies with the target protease. Whereas all seven P_2 variants of ACT reacted rapidly with trypsin, the nature of the P_2 residue markedly influenced the k_{on} value of the ACT variants with thrombin; a 970-fold difference in k_{on} values was observed between the fastest (ACT-PR) and the slowest (ACT-TR) inhibitors of thrombin. For thrombin, the importance of the P_2 residue can approach that of the P_1 residue in determining the rate of inhibition. With a P_2 proline, replacement of the P_1 arginine by leucine resulted in a 2580-fold decrease in k_{on}, and this effect is only 3 times larger than the difference observed between the k_{on} values for the ACT variants with a P_2 proline and threonine (Table 2).

The effects of the P_2 substitutions on the inhibition of APC were in general similar to those observed with trypsin; most of the changes in k_{on} caused by the P_2 replacements were less than 5-fold. The P_2 glycine mutant proved an exception to this generalization; the k_{on} value for ACT-GR with APC was 50-fold lower than that of the fastest variant (ACT-VR). This result is in accord with the poor inhibition of APC by antithrombin, which also has a P_2 glycine (Hermans & Stone, 1993). Phillips et al. (1994) also found that, for inhibition of APC, a P_2 glycine was significantly worse than the natural phenylalanine in protein C inhibitor. Similarly, replacement of the P_2 proline of P_1-Arg-α_1-antitrypsin by glycine causes a marked reduction in the k_{on} value for this serpin with APC (Hopkins et al., 1995). The effects of the P_2 replacements on the inhibition of factor Xa were akin to those seen with thrombin. While the k_{on} values for variants with alanine, proline, and glycine in P_2 varied by less than 5-fold, the values for the leucine, valine, and threonine variants were more than 100-fold lower than the value for the fastest inhibitor of factor Xa (ACT-GR).

Molecular Basis for P_2 Interactions with Trypsin and Thrombin. The effects of the P_2 substitutions on the inhibition of thrombin and trypsin can be readily interpreted in terms of interactions with the S_2 sites of these proteases. Interaction areas within the active site of thrombin have been defined by the crystal structures of thrombin in complex with D-Phe-Pro-ArgCH_2Cl and an analogue of fibrinopeptide A (Bode et al., 1989; Stubbs et al., 1992). In comparison with trypsin, thrombin contains a large insertion in a loop at the top of the active site, and this insertion restricts access to the active site and creates a particularly hydrophobic S_2 binding site. The P_2 proline of D-Phe-Pro-ArgCH_2Cl fills this hydrophobic pocket created by residues Tyr^{60A} and Trp^{60D} from the insertion loop as well as His^{57} and Leu^{99}. This S_2 site is well adapted for the binding of proline and most synthetic peptide inhibitors of thrombin have incorporated a proline (or proline analog) in the P_2 position (Tapparelli et al., 1993). In the fibrinopeptide A−thrombin structure, the P_2 valine occupies a similar position to the proline in D-Phe-Pro-ArgCH_2Cl but makes fewer contacts. The S_2 site of trypsin is much less restricted than that of thrombin (Huber et al., 1974; Marquart et al., 1983), and molecular modeling indicated that it would be possible to bind all seven P_2 residues tested to this site without any steric conflicts. In contrast, modeling studies with thrombin revealed that threonine or phenylalanine could not be accommodated readily by the S_2 subsite. The hydrophobic environment of this subsite was not suited to the hydrophilic nature of threonine, and phenylalanine was too large to fit into the pocket. While the other P_2 residues (alanine, valine, glycine, and leucine) could be accommodated within the S_2 subsite, the number of contacts was less than those observed with proline.

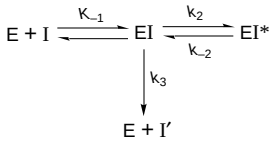
Interactions between ACT P_2 variants and thrombin were investigated further by examining their interactions with two previously characterized mutants of thrombin: des-PPW and E192Q. In the mutant des-PPW, the residues Pro^{60B}-Pro^{60C}-Trp^{60D} are deleted from the B-insertion loop of thrombin (Le Bonniec et al., 1993). Since this loop helps to form the S_2 site of thrombin, changes in the P_2 specificity of the mutant were expected. The effect of the deletion was in fact to decrease the specificity of thrombin such that des-PPW was more like trypsin in its specificity for P_2 residues. The k_{on} values for most of the mutants with des-PPW varied by less than 5-fold. The change in k_{on} caused by a particular substitution was in general about 10-fold less with des-PPW than that observed with thrombin; e.g., while the difference in the k_{on} values between ACT-PR and ACT-TR was 970-fold for thrombin, the difference was only 15-fold with des-PPW (Table 1). The net result of the smaller changes in k_{on} with des-PPW was that, whereas large differences in the k_{on} values between thrombin and des-PPW were observed with the fastest variants (100-fold for ACT-PR), the k_{on} values for some of the slower ones (ACT-TR in particular) were similar with des-PPW and thrombin.

The results obtained with the E192Q mutant provide further information on the role of Glu^{192} in regulating thrombin's specificity. It has previously been shown that Glu^{192} restricts thrombin activity with substrates that have acidic residues in the P_3 and P_3' positions (Le Bonniec & Esmon, 1991; Le Bonniec et al., 1992). Substitution of Glu^{192} with a glutamine, as found in trypsin and most other trypsin-like serine proteases, results in an enzyme (E192Q) that is more readily able to accommodate acidic residues in the P_3 and P_3' positions. Structural studies have also illustrated that residue 192 in trypsin- and chymotrypsin-like enzymes makes substantial contacts with positions P_2, P_1, and P_1' of inhibitors (Fujinaga et al., 1987; Steitz et al., 1969). The k_{on} values obtained for the ACT P_2 variants (and ACT-PL) with E192Q suggest that Glu^{192} may play a role in determining both the P_2 and P_1 preferences of thrombin. Increases in k_{on} values were seen for all ACT P_2 variants with E192Q relative to wild-type thrombin. A general

increase of about 10-fold was observed in k_{on} values with two notable exceptions: ACT-GR showed only a marginal improvement, whereas ACT-TR displayed a marked 53-fold increase in k_{on}. Molecular modeling based on the D-Phe-Pro-ArgCH_2−thrombin structure (Bode et al., 1989) indicated that a favorable hydrogen bond could possibly form between the hydroxyl of a threonine at position P_2 and the amide of Gln^{192} in E192Q. Substitution of Glu^{192} for glutamine also influenced the ability of thrombin to accommodate leucine in the P_1 position. The k_{on} value for ACT-PL was 7-fold higher with E192Q than with thrombin. This increase in k_{on} was, however, much smaller than that observed with α_1-antitrypsin, which has a P_1 methionine and P_2 proline. With this serpin, the k_{on} value with thrombin increased over 1000-fold as a result of replacing Glu^{192} by glutamine (Le Bonniec et al., 1995).

The P_2 Residue Can Affect the Partitioning between Substrate and Inhibition Pathways. Although the nature of the P_2 residue did not greatly influence the k_{on} for ACT variants with trypsin, the presence of a P_2 proline dramatically affected the stoichiometry of inhibition with this protease. The SI value of this ratio increased from less than 2 with most variants to over 100 with ACT-PR. SI values significantly greater than 1 have been observed for other serpins (Olson, 1985; Patston et al., 1991; Hopkins et al., 1993; Hood et al., 1994; Hermans et al., 1995) and they are consistent with a suicide substrate mechanism. In this mechanism (Scheme 2), an initial complex (EI) partitions between two alternative pathways: one leading to a stable protease−inhibitor complex (EI*) and one resulting in the production of cleaved serpin (I′).

Scheme 2



In this bifurcating reaction pathway, the initial enzyme−inhibitor complex forms a stable complex with a rate constant k_2 or a cleavage reaction occurs with a rate constant k_3. Under the conditions of the slow-binding assays, the measured association rate constant (k_{on}) will equal k_2/K_{-1}, where K_{-1} is the inhibition constant for the initial complex (Stone & Hermans, 1995). For this mechanism, Waley (1985) has defined k_3/k_2 as the partition ratio (r). This partition ratio represents the number of catalytic turnovers per inactivation event; the stoichiometry of inhibition (SI) is equal to ($1 + r$) (Waley, 1985). The value for k_{on} can be calculated either on the basis of the total concentration of serpin ($[I]_t$) or using the so-called concentration of "active" serpin derived from the stoichiometry of inhibition (SI). The concentration of "active" serpin equals $[I]_t/SI$ or $[I]_t/(1 + k_3/k_2)$. Waley (1985) has shown that the amount of cleaved inhibitor generated is equal to $r[E]_t$; i.e., the amount of cleaved inhibitor will depend on the total concentration of enzyme ($[E]_t$) and the partition ratio ($r = k_3/k_2$). For the determination of k_{on} values with thrombin and trypsin, $[I]_t$ was always much greater than $r[E]_t$; thus, depletion of the inhibitor due to the cleavage pathway could be ignored and the use of the total concentration of inhibitor for the calculation of k_{on} is the correct method. This point is illustrated in Figure 2 in which the reaction of ACT-PR with trypsin is simulated. For the

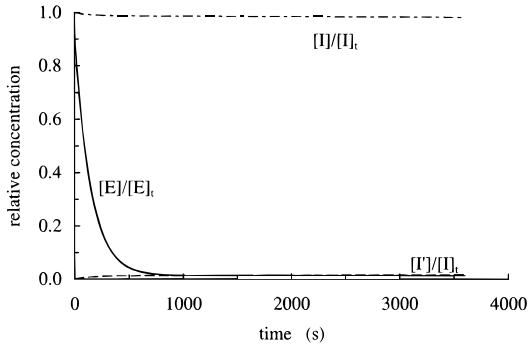


FIGURE 2: Simulation of the variation of the concentrations of protease and serpin species during the reaction of trypsin with ACT-PR. Data were simulated according to the differential equations describing the suicide substrate mechanism of Scheme 2 using numerical integration with a Runge−Kutta algorithm. Values of the rate constants were chosen such that k_{on} and r equaled 4×10^5 M^{-1} s^{-1} and 100, respectively. The concentrations of trypsin and ACT-PR were 0.025 nM and 0.2 μM, respectively. The value of $[S]/K_m$ was 10. The variation of the relative concentrations of free enzyme ($[E]/[E]_t$), cleaved serpin ($[I']/[I]_t$), and free serpin ($[I]/[I]_t$) are shown.

simulation, the values of the kinetic parameters (k_{on} and r) were those given in Tables 1 and 2, while the concentrations of trypsin and ACT-PR were among those used (0.025 nM and 0.2 μM, respectively). The data of Figure 2 demonstrate that during the course of the assay, the relative concentration of free enzyme ($[E]/[E]_t$) fell to near 0. However, the relative concentration of cleaved serpin ($[I']/[I]_t$) reached a plateau of less than 2%, i.e., the relative concentration of free (uncleaved) serpin ($[I]/[I]_t$) decreased by less than 2%. Although values of SI were not determined for the serpins with the other proteases tested, there is unlikely to be a significant decrease in the concentrations of free serpin with these proteases. The ratio $[I]_t/[E]_t$ was greater than 1000 in all cases. Since the concentration of cleaved serpin formed equals $r[E]_t$, even with an SI value of 100 (as observed for ACT-PR and trypsin), there would only be a 10% decrease in the concentration of serpin ($[I']/[I]_t = r[E]_t/[I]_t \leq 0.1$). Moreover, examination of the interactions of these proteases (factor Xa, APC, E192Q, and des-PPW) with the serpins by SDS−PAGE indicated that cleavage of the serpins did not occur to the same extent as with trypsin and ACT-PR with any of the reactions tested (data not shown).

From the SI value ($1 + k_3/k_2$) for ACT-PR with trypsin, it can be concluded that the rate constant (k_3) for the cleavage reaction was 100 times larger than the rate constant for stable complex formation (k_2). An increase in the partition ratio could be due to an increase in k_3 or a decrease in k_2. However, since the value of k_{on} ($= k_2/K_{-1}$) was not affected by the P_2 proline substitution, the increased value of the partition ratio is unlikely to be due to a decrease in the value of k_2; an increase in k_3 (the rate constant for cleavage) seems more likely. The rate of cleavage of tripeptidyl *p*-nitroanilide substrates with a P_2 proline by trypsin is not significantly faster than that of substrates with other P_2 residues (Lottenberg et al., 1983). Thus, a P_2 proline does not appear to increase the rate of cleavage of a peptide by trypsin *per se*. It can be speculated, however, that the P_2 proline has altered the conformation of the RSL such that it is more susceptible to cleavage. In the structure of a variant of ACT with P_3−P_3' residues of α_1-antitrypsin in the RSL, this loop exists in a distorted helical conformation (Wei et al., 1994). Part of this distortion is due to the presence of the helix-breaking

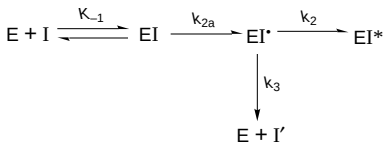
Table 3: Comparison of the Association Constants for Serpins with the Same P_2-P_1-P_1' Sequence[a]

serpin	k_{on} (M^{-1} s^{-1})			
	thrombin	APC	trypsin	factor Xa
antithrombin	1.1×10^4 [b]	1.4×10^{-1} [c]	1.4×10^5 [d]	3.1×10^3 [e]
ACT-GR	1.5×10^3	7.7×10^0	5.5×10^5	3.0×10^3
protease nexin 1	2.1×10^6 [f]	5.2×10^3 [c]	4.7×10^6 [f]	6.5×10^3 [g]
ACT-AR	1.8×10^3	1.9×10^4	4.1×10^5	2.1×10^1
P_1-Arg-α_1-antitrypsin	3.1×10^5 [h]	7.0×10^4 [c]	4.2×10^5 [c]	1.9×10^4 [i]
ACT-PR	3.3×10^4	2.0×10^2	4.4×10^5	6.8×10^2
protein C inhibitor	3.3×10^4 [c]	8.0×10^2 [c]	2.2×10^4 [j]	2.6×10^3 [k]
ACT-FR	2.2×10^2	7.5×10^2	3.1×10^5	7.4×10^1

[a] Values for the ACT P_2 variants were taken from Table 2; other values were taken from the sources listed. [b] Wallace et al. (1989). [c] Hermans and Stone (1993). [d] Holmes et al. (1987). [e] Jordan et al. (1980). [f] Stone et al. (1987). [g] J. M., Hermans, S. R. Stone, unpublished results. [h] Jallat et al. (1986). [i] George and Carrell (1986). [j] Hermans et al. (1994). [k] Cooper and Church (1994).

proline in the P_2 position. Thus, it seems possible that the observed increase rate of cleavage is due to a distortion in the structure of the RSL caused by the proline, which in turn makes the loop more susceptible to cleavage by trypsin. It should be noted, however, the influence of a P_2 proline on the rate of cleavage by trypsin depends on the sequence of the loop; α_1-antitrypsin, which also contains a P_2 proline, is not cleaved by trypsin to an appreciable extent (Hopkins et al., 1993).

Scheme 3



The above analysis of the data for the P_2 proline variants with trypsin was based on the suicide substrate mechanism of Scheme 2. Other workers (e.g., Hood et al., 1994) have based their analysis on a mechanism which includes an extra step before the partitioning of the inhibition and substrate pathways (Scheme 3). For this mechanism, the apparent second-order rate constant for the formation of the stable EI* complex (defined as k_{on} in the present study) will be $k_{2a}/(K_{-1}(1 + k_3/k_2))$. The apparent second-order rate constant for the formation of the branch-point complex (EI•) will be k_{2a}/K_{-1}, or k_{on}SI. Analysis of the data for ACT-PR with trypsin according to this mechanism suggests that at least two rate constants have been altered by the P_2 proline mutation. The mutation has increased the SI value by either increasing the magnitude of k_3 or decreasing that of k_2. In addition, the apparent second-order rate constant for the formation of the branch-point complex (k_{2a}/K_{-1}) has been increased 100-fold by the P_2 proline mutation. Thus, on the basis of simplicity the mechanism of Scheme 2, in which partitioning occurs after the binding step, must be preferred, since the value of only one rate constant (k_3) must be altered to explain the data in the terms of that mechanism. Other circumstantial evidence also argues against the mechanism of Scheme 3. According to this mechanism, the value of k_{2a}/K_{-1} ($= k_{on}$SI) for ACT-PR with trypsin would be at least 30-fold higher than those other P_2 variants, whose values were all more or less equivalent, and the value of k_{2a}/K_{-1} for ACT-PR would be 100-fold higher than that calculated for P_1-Arg-α_1-antitrypsin, which has the same P_2-P_1-P_1' sequence (Table 3).

Serpin Specificity and the P_2-P_1-P_1' Sequence. Comparison of the results obtained in the present study with the k_{on} values for natural serpins gives some indication of the importance of residues other than P_2, P_1, and P_1' in regulating specificity. The P_2-P_1-P_1' sequences of antithrombin (Gly-Arg-Ser), protease nexin 1 (Ala-Arg-Ser), P_1-Arg-α_1-antitrypsin (Pro-Arg-Ser), and protein C inhibitor (Phe-Arg-Ser) have been reproduced in ACT-GR, ACT-AR, ACT-PR, and ACT-FR, respectively, and the k_{on} values for the ACT variants are compared with those for the natural serpins in Table 3. For trypsin, the k_{on} values of the natural serpins were comparable to those of the ACT variants with the same P_2-P_1-P_1' sequence, which suggests that residues outside of this sequence in the natural serpins play a minor role in regulating the inhibition of trypsin. Within this sequence, the P_1 residue appears to be a major determinant of serpin specificity with respect to trypsin; a 600-fold decrease in k_{on} was observed upon replacement of the P_1 arginine of ACT-PR by leucine (Table 2). However, trypsin is able to accommodate diverse residues even in the P_1 position of serpins; a difference of less than 10-fold in k_{on} values is observed between natural α_1-antitrypsin with a P_1 methionine and P_1-Arg-α_1-antitrypsin (Travis et al., 1985).

For the interactions of serpins with proteases other than trypsin, residues beyond the P_2-P_1-P_1' sequence appear to be important in either promoting or restricting inhibition. Although the inhibitory activity of ACT-GR was similar to that of antithrombin with thrombin and factor Xa, the k_{on} value of this ACT variant was 55-fold higher than that of antithrombin with APC, suggesting the presence of other residues in antithrombin that restrict the inhibition of this protease. Nevertheless, the inhibitory properties of ACT-GR, particularly with respect to APC inhibition, were much more akin to those of antithrombin than those of an α_1-antitrypsin variant with the same P_2-P_1-P_1' sequence (Hopkins et al., 1995). For the α_1-antitrypsin variant with the P_2-P_1-P_1' sequence (Gly-Arg-Ser) of antithrombin, a k_{on} value of 4.3×10^3 M^{-1} s^{-1} was observed with APC, which is 10^4-fold higher than the corresponding value for antithrombin and 10^2-fold higher than that of ACT-GR. The similar kinetic properties of ACT-GR and antithrombin are probably at least partly due to the extended homology in the RSL between ACT-GR and antithrombin; six residues in the P_1-P_8 sequence are identical between antithrombin and ACT-GR. A large difference between P_1-Arg-α_1-antitrypsin and ACT-PR was also observed with APC; the k_{on} value for P_1-Arg-α_1-antitrypsin with APC is 350-fold higher than that of ACT-PR. This result suggests additional sequences in α_1-antitrypsin promote its interaction with APC, and this may have some relevance to the observed inhibition of this protease by α_1-antitrypsin *in vivo* despite the P_1

methionine of this serpin (Heeb & Griffin, 1988; Heeb et al., 1990).

For protease nexin 1, the results suggest the presence of residues beyond the P_2-P_1-P_1' sequence that promote the inhibition of thrombin and factor Xa. Both these proteases were inhibited more than 300 times faster by protease nexin 1 than by ACT-AR. Although the inhibitory properties of ACT-FR with respect to APC were identical to those of protein C inhibitor, the k_{on} value of this variant with thrombin was 150-fold lower than that of protein C inhibitor, suggesting that other sequences may also be important in regulating the inhibitory specificity of this serpin.

Conclusion. The P_2 residue of serpins appears to play an important role in regulating the specificity of these inhibitors. The P_2 residue not only modulates the rate at which the serpin reacts with a particular protease but also can influence the partitioning of the serpin between inhibition and cleavage pathways. For a number of serpin−protease interactions, specificity can be largely accounted for by the nature of the P_2-P_1-P_1' sequence. For other serpin−protease pairs, however, residues beyond the P_2-P_1-P_1' sequence are important in either accelerating (e.g., protease nexin 1−thrombin) or retarding (e.g., antithrombin−APC) the reaction.

REFERENCES

Albert, B., & Herrick, G. (1971) *Methods Enzymol. 21*, 198−217.

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8*, 3467−3475.

Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177−2185.

Chase, T., Jr., & Shaw, E. (1970) *Methods Enzymol. 19*, 20−27.

Cooper, S. T., & Church, F. C. (1994) *Biochim. Biophys. Acta 1246*, 29−33.

Derechin, V. M., Blinder, M. A., & Tollefsen, D. M. (1990) *J. Biol. Chem. 265*, 5623−5628.

Duggleby, R. G., & Morrison, J. F. (1978) *Biochim. Biophys. Acta 526*, 398−409.

Eldering, E., Huijbregts, C. C. M., Lubbers, Y. T. P., Longstaff, C., & Hack, C. E. (1992) *J. Biol. Chem. 267*, 7013−7020.

Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol. 195*, 397−418.

George, P. M., & Carrell, R. W. (1986) in *Design of Enzyme Inhibitors as Drugs* (Sandler, M., & Smith, H. J., Eds.) pp 581−596, Oxford University Press, Oxford, England.

Heeb, M. J., & Griffin, J. H. (1988) *J. Biol. Chem. 263*, 11613−11616.

Heeb, M. J., Bischoff, R., Courtney, M., & Griffin, J. H. (1990) *J. Biol. Chem. 265*, 2365−2369.

Hermans, J. M., & Stone, S. R. (1993) *Biochem. J. 295*, 239−245.

Hermans, J. M., Jones, R., & Stone, S. R. (1994) *Biochemistry 33*, 5440−5444.

Hermans, J. M., Jones, R., Monard, D., & Stone, S. R. (1995) *Biochemistry 34*, 3678−3685.

Holmes, W. E., Lijnen, H. R., & Collen, D. (1987) *Biochemistry 26*, 5133.

Hood, D. B., Hungtindon, J. A., & Gettins, P. G. W. (1994) *Biochemistry 33*, 8538−8547.

Hopkins, P. C. R., Carrell, R. W., & Stone, S. R. (1993) *Biochemistry 32*, 7650−7657.

Hopkins, P. C. R., Crowther, D. C., Carrell, R. W., & Stone, S. R. (1995) *J. Biol. Chem. 270*, 11866−11871.

Huber, R., & Carrell, R. W. (1989) *Biochemistry 28*, 8951−8966.

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steiggemann, W. (1974) *J. Mol. Biol. 89*, 73−101.

Jallat, S., Carvallo, D., Tessier, L. H., Roecklin, D., Roitsch, C., Ogushi, F., Crystal, R. G., & Courtney, M. (1986) *Protein Eng. 1*, 29−35.

Jordan, R. E., Oosta, G. M., Gardner, W. T., & Rosenberg, R. D. (1980) *J. Biol. Chem. 255*, 10081−10090.

Kunkel, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Le Bonniec, B. F., & Esmon, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7371−7375.

Le Bonniec, B. F., Guinto, E. R., & Esmon, C. T. (1992) *J. Biol. Chem. 267*, 6970−6976.

Le Bonniec, B. F., Guinto, E. R., MacGillivray, R. T. A., Stone, S. R., & Esmon, C. T. (1993) *J. Biol. Chem. 268*, 19055−19061.

Le Bonniec, B. F., Guinto, E. R., & Stone, S. R. (1995) *Biochemistry 34*, 12241−12248.

Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 539−557.

Marquart, M., Walter, J., Deisenhofer, J., Bode, W., & Huber, R. (1983) *Acta Crystallogr. 39*, 480−490.

Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102−105.

Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201−301.

Olson, S. T. (1985) *J. Biol. Chem. 260*, 10153−10160.

Owen, M. C., Brennan, S. O., Lewis, J. H., & Carrell, R. W. (1983) *New Engl. J. Med. 309*, 694−698.

Owen, M. C., George, P. M., Lane, D. A., & Boswell, D. R. (1991) *FEBS Lett. 280*, 216−220.

Patston, P. A., Gettins, P., Beecham, J., & Schapira, M. (1991) *Biochemistry 30*, 8876−8882.

Phillips, J. E., Cooper, S. T., Potter, E. E., & Church, F. C. (1994) *J. Biol. Chem. 269*, 16696−16700.

Potempa, J., Korzus, E., & Travis, J. (1994) *J. Biol. Chem. 269*, 15957−15960.

Rubin, H., Wang, Z.-M., Nickbarg, E. B., McLarney, S., Naidoo, N., Schoenberger, O. L., Johnson, J. L., & Cooperman, B. S. (1990) *J. Biol. Chem. 265*, 1199−1207.

Schapira, M., Ramus, M.-A., Brunner, H. R., Jallat, S., Carvallo, D., Roitsch, C., & Courtney, M. (1987) *J. Clin. Invest. 80*, 582−585.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.

Sheffield, W. P., & Blajchman, M. A. (1994) *FEBS Lett. 339*, 147−150.

Sherman, P. M., Lawrence, D. A., Yang, A. Y., Vandenberg, E. T., Paielli, D., Olsen, S. T., Shore, J. D., & Ginsberg, D. (1992) *J. Biol. Chem. 267*, 7588−7595.

Skriver, K., Radziejewska, E., Silbermann, J. A., Donaldson, V. H., & Bock, S. C. (1989) *J. Biol. Chem. 264*, 9216−9221.

Steitz, T. A., Henderson, R., & Blow, D. M. (1969) *J. Mol. Biol. 46*, 337−348.

Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622−4628.

Stone, S. R., & Hermans, J. M. (1995) *Biochemistry 34*, 5164−5172.

Stone, S. R., Nick, H., Hofsteenge, J., & Monard, D. (1987) *Arch. Biochem. Biophys. 252*, 237−244.

Stubbs, M. T., Oschkinat, H., Mayr, I., Huber, R., Angliker, H., Stone, S. R., & Bode, W. (1992) *Eur. J. Biochem. 206*, 187−195.

Tapparelli, C., Metternich, R., Ehrhardt, C., & Cook, N. S. (1993) *Trends Pharmacol. Sci. 14*, 366−376.

Travis, J., & Morii, M. (1981) *Methods Enzymol. 80*, 765−770.

Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655−709.

Travis, J., Owen, M., George, P., Carrell, R., Rosenberg, S., Hallewell, R. A., & Barr, P. J. (1985) *J. Biol. Chem. 260*, 4384−4389.

Waley, S. G. (1985) *Biochem. J. 227*, 843−849.

Wallace, A., Rovelli, G., Hofsteenge, J., & Stone, S. R. (1989) *Biochem. J. 257*, 191−196.

Wei, A., Rubin, H., Cooperman, B. S., & Christianson, D. W. (1994) *Nature Struct. Biol. 1*, 251−258.

York, J. D., Li, P., & Gardell, S. J. (1991) *J. Biol. Chem. 266*, 8495−8500.

BI952717I