**Communication**

**Serpin-Protease Complexes Are Trapped as Stable Acyl-Enzyme Intermediates**

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The serine protease inhibitors of the serpin family are an unusual group of proteins thought to have metastable native structures. Functionally, they are unique among polypeptide protease inhibitors, although their precise mechanism of action remains controversial. Conflicting results from previous studies have suggested that the stable serpin-protease complex is trapped in either a tight Michaelis-like structure, a tetrahedral intermediate, or an acyl-enzyme. In this report we show that, upon association with a target protease, the serpin reactive-center loop (RCL) is cleaved resulting in formation of an acyl-enzyme intermediate. This cleavage is coupled to rapid movement of the RCL into the body of the protein bringing the inhibitor closer to its lowest free energy state. From these data we suggest a model for serpin action in which the drive toward the lowest free energy state results in trapping of the protease-inhibitor complex as an acyl-enzyme intermediate.

The serpins are a large family of proteins which includes most of the protease inhibitors found in blood, as well as other proteins with unrelated or unknown functions (1). Serpins act as "suicide inhibitors" that react only once with their cognate proteases, forming an SDS-stable complex. Current models of serpin-protease complexes are controversial (15–21, 28). In the late 1970s, it was reported that serpins were unlike other tight binding protease inhibitors and formed covalent ester linkages with enzymes (15). However, these conclusions were based on SDS-PAGE analysis of denatured complexes leaving the nature of the native complex open to question. Later investigations suggested that the native serpin-protease complex may be reversible and therefore could not be covalent but instead might form a Michaelis-like complex similar to the tight binding inhibitors of the Kunitz and Kazal families (16). Finally, a recent NMR study suggests a stable tetrahedral configuration (19). To distinguish between these alternative structural models, we developed methods to monitor the position of the RCL in the inhibitor-enzyme complex and to determine the chemical nature of the association between serpins and their target proteases.

**EXPERIMENTAL PROCEDURES**

Materials—PAI-1 mutants containing Cys substitutions at either the P9 position (Ser-338) or the P1′ (Met-347) of the RCL were constructed by site-directed mutagenesis as described (14). Both mutants were purified and labeled with the environmentally sensitive probe NBD (Molecular Probes) as described (21). The labeled mutants retained full inhibitory activity toward both urokinase (uPA) and tissue-type plasminogen activator (tPA), with second order rate constants for inhibition of >10^4 mM^-1 s^-1 in all cases. Recombinant high molecular weight uPA was a generous gift of Dr. J. Henkin of Abbott Laboratories, and two-chain tPA was prepared from Activase (Genentech) as described previously (22). Purine pancreatic elastase was from Elastin Products (Owensville, MO), and human α,α,α,α-AT was from Athens Research and Technology (Athens, GA). 125I-labeled Bolton-Hunter reagent (22) (moniodinated) was from DuPont NEN, and N-hydroxysuccinimide acetic acid (NHS) was from Sigma.

Stopped-flow Fluorescence Analysis—Stopped-flow fluorimetry was performed on both PAI-1 NBD derivatives as described (21). PAI-1 concentrations were 0.25 μM for the P1-NBD derivative and 0.1 μM for the P1′-NBD derivative and 0.1 μM for

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Currently, the role of loop mobility in serpin function and the structure of the serpin-protease complex are controversial (15–21, 28). In the late 1970s, it was reported that serpins were unlike other tight binding protease inhibitors and formed covalent ester linkages with enzymes (15). However, these conclusions were based on SDS-PAGE analysis of denatured complexes leaving the nature of the native complex open to question. Later investigations suggested that the native serpin-protease complex may be reversible and therefore could not be covalent but instead might form a Michaelis-like complex similar to the tight binding inhibitors of the Kunitz and Kazal families (16). Finally, a recent NMR study suggests a stable tetrahedral configuration (19). To distinguish between these alternative structural models, we developed methods to monitor the position of the RCL in the inhibitor-enzyme complex and to determine the chemical nature of the association between serpins and their target proteases.
the P9-NBD derivative. The tPA concentration ranged from 0.15 μM to 6.0 μM.

Bolton-Hunter Labeling—Wild type PAI-1 or substrate PAI-1 (Thr-333 → Arg(7)) at a concentration of 1 μM was incubated ± 1 μM uPA or tPA in 50 mM sodium phosphate, pH 7.6, 150 mM NaCl at 23 °C for 30 min, followed by the addition of 125I-labeled Bolton-Hunter reagent to 100 nM. The samples were incubated an additional 30 min followed by the addition of 1/10 volume of stop solution (1 M glycine, 50 mM Tris, 150 mM NaCl, pH 7.75) and incubation continued for an additional 30 min. The labeled proteins were then separated from free 125I-Bolton-Hunter by precipitation with 50% saturated ammonium sulfate for 30 min at 4 °C followed by centrifugation at 14,000 × g for 20 min. The supernatant, containing free 125I-Bolton-Hunter, was discarded, and the pellets were washed twice with 50% saturated ammonium sulfate. The pellets were resuspended in 20 μl of 1% SDS, 10 mM Tris, pH 7.4, 1 mM EDTA, and −250,000 cpm of each sample was subjected to SDS-PAGE on a 20% homogeneous gel (PhastGel, Pharmacia Biotech Inc.) followed by staining with Coomassie Brilliant Blue and autoradiography.

Blocking of Free Amino-terminal Residues—Inhibitor (1 μM) was incubated with enzyme (1 μM) for 30 min at 23 °C in 50 mM sodium phosphate, pH 7.6, 150 mM NaCl, after which NHS was added to either 100 μM or 1 mM. The samples were incubated an additional 30 min followed by the addition of 1/10 volume of 1 μM Tris, pH 7.5, and continued incubation for 30 min. The Tris was then removed by ultrafiltration and washing with distilled water in a Prospin column (Applied Biosystems) prior to automated amino-terminal sequence analysis using Edman chemistry (Applied Biosystems model 473A). Pretreatment of PAI-1 or tPA with NHS at both NHS concentrations tested did not significantly affect the activity of either protein, indicating that, under these conditions, NHS treatment alone does not result in protein denaturation.

Molecular Modeling—The model for active PAI-1 has been described previously (23). The model for cleaved PAI-1 was generated using Quanta (Burlington, MA) from the coordinates of latent PAI-1 generously provided by Dr. E. Goldsmith and from the coordinates of cleaved αTAT obtained from the Brookhaven data base. The root mean square difference of the Cα trace with latent PAI-1 is <0.04 Å and 2.2 Å with cleaved αTAT.

RESULTS AND DISCUSSION

Stopped-flow Fluorescence Analysis—In the first series of experiments, site-directed mutants of the serpin PAI-1 were constructed with Cys residues at either the P9 or the P1 position of the RCL. Each mutant was then labeled with the fluorescent probe NBD. This probe shows a large enhancement in fluorescence when moved from an aqueous environment to a hydrophobic milieu. Both mutants were then reacted with tissue-type plasminogen activator (TPA) in a stopped-flow fluorimeter, and these results are shown in Fig. 1. Reaction of the 9-NBD PAI-1 with TPA resulted in a large and rapid enhancement of the relative fluorescence (Fig. 1) together with a 13 nm blue spectral shift (data not shown), consistent with our previous data (21). The extent of this change is nearly identical with that observed during the transition to the latent conformation (21) and is consistent with insertion of the RCL into β-sheet A and the resultant burying of the P9 residue beneath α-helix F. The observed rates of loop insertion over a range of tPA concentrations are shown in the inset of Fig. 1. These data yield a limiting rate constant for this reaction of −4 s⁻¹ (τ₁/₂ for insertion of ~250 ms). In contrast, reaction of the P9-NBD PAI-1 with TPA resulted in a 30% decrease in relative fluorescence occurring at approximately the same rate (Fig. 1). This quench indicates that, unlike the P9 position of the RCL, the P1 side chain is exposed to a more hydrophilic environment upon reaction with TPA. Although such a shift in position could result from a minor conformational change in the RCL, this explanation seems unlikely given the close association of the serpin and protease via the directly adjacent P1 Arg residue of PAI-1 and the S1 subsite of TPA. Alternatively, cleavage of the P9-NBD PAI-1 RCL by TPA between the P1 and P9 residues could permit the NBD reporter group to move away from the enzyme into a more aqueous environment. In either case, the limiting rate of the reaction, calculated from the data in the inset to Fig. 1 is similar to that observed with the P9-NBD mutant (~8 s⁻¹ versus 4 s⁻¹), indicating that changes at the P1 site must occur either immediately preceding or concurrent with loop insertion. Since the maximum changes in fluorescence are stable over time (data not shown), the reaction being monitored in the stopped flow is proceeding to completion and thus represents formation of the stable inhibitor-enzyme complex and not a transient or intermediate reaction.

Bolton-Hunter Labeling of Free Amines—To distinguish between cleavage of the P1-P1' peptide bond versus solely a conformational change in the intact RCL, the PAI-1-TPA complex was reacted with the amino-specific 125I-Bolton-Hunter reagent (22). Two different plasminogen activators were used, TPA and uPA. These experiments were conducted with trace

![Fig. 1. Stopped-flow kinetic analysis of the change in the relative fluorescence of 0.25 μM P1-NBD PAI-1 (●) or 0.1 μM P9-NBD PAI-1 (×) reacting with 2 μM TPA. The inset shows the pseudo-first order rate constants (kobs) for P1-NBD PAI-1 (●) and P9-NBD PAI-1 (×) determined from the change in NBD fluorescence versus TPA concentration. (The first 50 ms of each trace is not shown because of injection noise.)](image.png)

![Fig. 2. SDS-PAGE analysis of 125I-Bolton-Hunter reagent-treated PAI-1 ± uPA or TPA. A, 20% homogeneous gel stained with Coomassie Blue; lane 1, wild type PAI-1 only; lane 2, wild type PAI-1 + uPA; lane 3, wild type PAI-1 + tPA; lane 4, uPA only; lane 5, tPA only; lane 6, substrate PAI-1 only; lane 7, substrate PAI-1 + uPA; lane 8, substrate PAI-1 + tPA. B, autoradiography of the gel in A. The numbers at the left indicate the position of molecular mass standard proteins, and the arrow marks the position of the labeled carboxyl-terminal peptide.)](image.png)
Serpin-Protease Complexes Are Acyl-Enzyme Intermediates

The first column (NHS) gives the final concentration of NHS used in the pretreatment step. Yields are given in picomoles and represent the average yield of the first 5 residues in each sequence. Blocked, percent reduction in average yield relative to no NHS treatment; uPAc, uPA light chain; uPAhc, uPA heavy chain; PAI-1 N, the natural amino terminus of PAI-1; PAI-1 P, the newly generated PAI-1 carboxyl-terminal peptide beginning with the P1 residue.

| NHS | uPAc| Blocked | % | pmol | | uPAhc| Blocked | % | pmol | | PAI-1 N| Blocked | % | pmol | | PAI-1 P| Blocked | % | pmol |
|-----|-----|---------|---|------| |       |         |   |      | |       |         |   |      | |       |         |   |      |
| μM | pmol | % | pmol | | pmol | % | pmol | % | pmol | % |
| 0   | 39 (± 5.6) | 0 | 53 (± 6.2) | 0 | 37 (± 3.3) | 0 | 34 (± 2.4) | 0 |
| 100 | 26 (± 3.6) | 24 | 49 (± 4.6) | 8 | 26 (± 2.8) | 32 | 24 (± 2.2) | 29 |
| 1000| 4.0 (± 0.7) | 88 | 17 (± 4.4) | 68 | 3.1 (± 0.9) | 92 | 2.5 (± 1.0) | 93 |

* Picomole yields are ± S.E.

## Table II

Complex formation, NHS treatment, and analysis were exactly as for PAI-1-uPA complexes in Table I. NHS, final concentration of NHS used in the pretreatment step. Yields and Blocked were determined as in Table I. α1AT, the natural amino terminus of α1AT; α1AT, the newly generated α1AT carboxyl-terminal peptide beginning with the P1 residue.

| NHS | Elastase | Blocked | % | pmol | | u1AT N | Blocked | % | pmol | | u1AT P | Blocked | % | pmol |
|-----|---------|---------|---|------| |       |         |   |      | |       |         |   |      |
| μM | pmol | % | pmol | | pmol | % | pmol | % |
| 0   | 40 (± 3.6) | 0 | 46 (± 6.6) | 0 | 36 (± 5.5) | 0 |
| 100 | 27 (± 2.5) | 33 | 34 (± 4.4) | 27 | 21 (± 3.5) | 43 |
| 1000| 3.6 (± 0.8) | 91 | 3.9 (± 0.5) | 92 | 2.3 (± 0.6) | 94 |

* Picomole yields are ± S.E.

amounts of 125I-Bolton-Hunter reagent under non-denaturing conditions, followed by treatment of the unreacted label with glycine and removal prior to SDS-PAGE analysis. This procedure should report the presence of a cleaved RCL in the complex by the appearance of a novel labeled peptide fragment of the correct size. Furthermore, since the unreacted Bolton-Hunter reagent was blocked with glycine and removed before the samples were denatured by exposure to SDS, any labeled peptide must have been formed while the complexes were in their native state. Although all accessible amines could potentially be labeled, including e-NH2 groups of internal Lys residues, the only position that can incorporate label in the PAI-1 RCL carboxyl-terminal peptide would be its amino terminus, since PAI-1 contains no Lys residues in the 33-residue peptide produced by cleavage of the P1-P1 bond. SDS-PAGE analysis of the labeled complexes shown in Fig. 2 demonstrated a unique ~3.0-kDa band with both PAI-1-PA complexes which was not present with PAI-1 or either PA alone (B). The observed mobility of this novel peptide is consistent with the predicted molecular mass (3.8 kDa) of the PAI-1 carboxyl-terminal peptide. As a positive control for cleavage of the RCL and the labeling efficiency of the C-terminal peptide, we also tested a mutant PAI-1 that we have previously shown is a pure substrate for plasminogen activators and is completely cleaved at its RCL P1-P1’ peptide bond (7). Consistent with previous observations, the mutant PAI-1 fails to form stable complexes with either PA and is instead completely cleaved (Fig. 2A, compare lanes 2 and 3 with lanes 7 and 8). Furthermore, a labeled peptide identical with that observed with wild type PAI-1-PA complexes is also seen (Fig. 2B, compare lanes 2 and 3 with lanes 7 and 8). This similar efficiency of peptide labeling for the mutant PAI-1 cleaved by either uPA or tPA compared to wild type PAI-1 in association with each enzyme indicates that the RCL within the stable serpin-protease complex is cleaved and suggests that this cleavage is complete.

Quantitation of Free Amino-terminal Residues—To confirm complete cleavage of the PAI-1-PA complex and exclude substrate behavior by a subset of the inhibitor molecules, the extent of RCL cleavage was directly quantitated by microsequencing of the PAI-1-uPA complex. Since complex denaturation during the sequencing reaction could potentially induce cleavage, the extent of cleavage in native complexes was determined by a subtractive method. PAI-1-uPA complexes were first reacted with the amino-specific reagent NHS under non-denaturing, physiological conditions. This compound is similar to Bolton-Hunter reagent in its reactivity and covalently binds to free amines. Treatment of PAI-1-uPA complexes with NHS should therefore block available amino termini in a dose-dependent manner. The excess NHS was then reacted with Tris and removed by ultrafiltration prior to direct amino-terminal sequence analysis of remaining unreacted amino termini. This analysis is quantitative, and the relative reactivity of natural amino termini from both the inhibitor and protease serve as internal controls for NHS reactivity and sequencing efficiency. The results of this analysis are shown in Table I. The yield of RCL peptide amino terminus at each dose of NHS is very similar to those of the natural PAI-1 and uPA amino termini. Thus, the RCL peptide amino terminus generated upon complex formation is as reactive as the natural amino termini and the enzyme is very likely to be fully cleaved and exposed consistent with the quenched fluorescence of the P1'-NBD PAI-1 and the 125I labeling results. Interestingly, the least NHS-reactive amino terminus tested is that of the uPA heavy chain (uPA hormone, Table I), a relatively hydrophobic sequence Ile-Ile-Gly-Gly which is likely to be oriented toward the interior of the molecule (24). The latter observation suggests that this approach is quite sensitive to amino termini solvent accessibility, further supporting the conclusion that the PAI-1 RCL must be completely cleaved when in complex with uPA. Similar results were also obtained with PAI-1-tPA complexes (data not shown).

To test the general relevance of these observations for other serpin-protease complexes, the α1AT-elastase complex was also treated with NHS and subjected to microsequencing. The results shown in Table II are similar to the data obtained with the PAI-1-uPA complexes (Table I), demonstrating that α1AT is also cleaved in its RCL when in complex with elastase. Taken together with the known stability of these complexes to SDS-PAGE, these observations strongly suggest that the serpin-protease complex is trapped in the form of a covalent acyl-enzyme intermediate. Serpin inhibition appears to be a two-step process with an initial reversible encounter complex followed by formation of an apparently irreversible stable com-
to the inhibitor. Such a rapid shift in the relative positions of the two molecules, centered at the enzyme's active site, might sufficiently distort the active site geometry to prevent efficient deacylation and thus trap the complex. Alternatively, the new position of the acylated protease's active site may prevent the water necessary for deacylation from entering the active site. This model is consistent with our previous results demonstrating that RCL insertion is not required for protease binding but is necessary for stable inhibition (7) as well as the observation that only an active enzyme can induce RCL insertion. We suggest that native serpin structures are kinetically trapped in a conformation which is not their most stable structure, and that the stored drive toward a lower energy structure results in trapping of the protease-inhibitor complex in the acyl-enzyme intermediate form.

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Note Added in Proof—Similar results, indicating that the serpin-protease complex is in the form of an acyl-intermediate, was recently presented by Wilczynska et al. (M. Wilczynska, M. Fa, P.-I. Olhsson, and T. Ny, submitted for publication) at the 5th International Workshop on the Molecular and Cellular Biology of Plasminogen Activation in Hämeenlinna, Finland.

REFERENCES

1. Huber, R., and Carrell, R. W. (1989) Biochemistry 28, 8951–8966
2. Carrell, R. W., Pemberton, P. A., and Boswell, D. R. (1987) Cold Spring Harbor Symp. Quant. Biol. 52, 527–535
3. We, A., Rubin, H., Cooperman, B. S., and Christianson, D. W. (1994) Nature Struct. Biol. 1, 251–258
4. Carrell, R. W., Stan, P. E., Ferni, G., and Wardell, M. R. (1994) Structure 2, 257–270
5. Schreuder, H. A., de Boer, B., Mulders, J., Theunissen, H. J. M., Groenenhuis, P. D. J., and Hol, W. G. J. (1994) Nature Struct. Biol. 1, 48–54
6. Carrell, R. W., Evans, D. L., and Stein, P. E. (1991) Nature 353, 576–578
7. Lawrence, D. A., Olson, S. T., Palaniappan, S., and Ginsburg, D. (1994) J. Biol. Chem. 269, 27657–27662
8. Stein, P. E., and Carrell, R. W. (1995) Nature Struct. Biol. 2, 96–113
9. Loebermann, H., Tokuoka, R., Diesenhofer, J., and Huber, R. (1984) J. Mol. Biol. 177, 531–557
10. Motonobu, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D., and Goldsmith, E. J. (1992) Nature 355, 270–273
11. Carrell, R. W., and Owen, M. C. (1985) Nature 317, 730–732
12. Gettinis, P., and Harten, B. (1988) Biochemistry 27, 3634–3639
13. Bruch, M., Weiss, V., and Engel, J. (1988) J. Biol. Chem. 263, 16626–16630
14. Lawrence, D. A., Olson, S. T., Palaniappan, S., and Ginsburg, D. (1994) Biochemistry 33, 3643–3648
15. Cohen, A. B., Gruenke, L. D., Craig, J. C., and Geczy, D. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4311–4314
16. Shieh, B. H., Potempa, J., and Travis, J. (1989) J. Biol. Chem. 264, 13420–13423
17. Lawrence, D. A., Strandberg, L., Ericson, J., and Ny, T. (1990) J. Biol. Chem. 265, 20293–20301
18. Longstaff, C., and Gaffney, P. J. (1991) Biochemistry 30, 979–986
19. Matheson, N. R., and van Halbeek, H., and Travis, J. (1991) J. Biol. Chem. 266, 13489–13491
20. Christensen, S., Valnickova, Z., Togerson, I. B., Pizzio, S. V., Nielsen, H. R., Roepstorff, P., and Engvild, J. J. (1995) J. Biol. Chem. 270, 14859–14862
21. Shore, J. D., Day, D. E., Francis-Chmura, A. M., Verhamme, I., Kvasman, J., Lawrence, D. A., and Ginsburg, D. (1995) J. Biol. Chem. 270, 5395–5398
22. Bolton, A. E., and Hunter, W. M. (1973) Biochemistry 12, 333–339
23. Berkenpas, M., B., Lawrence, D. A., and Ginsburg, D. (1995) EMBO J. 14, 2969–2977
24. Fresh, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, H. H. (1970) Biochemistry 9, 1977–2009
25. Cooperman, B. S., Stavvidi, E., Nickberg, E., Rescoria, E., Schechter, N. M., and Rubin, H. (1993) J. Biol. Chem. 268, 23636–23625
26. Kraut, J. (1977) Annu. Rev. Biochem. 46, 331–359
27. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162
28. Fa, M., Aleshkov, S., Strandberg, L., Karonin, J. J., Johansson, L. B.-Å., and Ny, T. (1994) Fibrinolysis 8, Suppl. 1, p. 17, (Abstr. 48)