The Reaction of Serpins with Proteinases Involves Important Enthalpy Changes

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ABSTRACT: When active serpins are proteolytically inactivated in a substrate-like reaction, they undergo an important structural transition with a resultant increase in their conformational stability. We have used microcalorimetry to show that this conformational alteration is accompanied by an important enthalpy change. For instance, the cleavage of α_1-proteinase inhibitor by *Pseudomonas aeruginosa* elastase, *Staphylococcus aureus* V8 proteinase, or papain and that of antithrombin by leukocyte elastase are characterized by large enthalpy changes ($\Delta H = -53$ to -63 kcal mol^{-1}). The former reaction also has a large and negative heat capacity ($\Delta C_p = -566$ cal K^{-1} mol^{-1}). In contrast, serpins release significantly less heat when they act as proteinase inhibitors. For example, the inhibition of pancreatic elastase, leukocyte elastase, and pancreatic chymotrypsin by α_1-proteinase inhibitor and that of pancreatic trypsin and coagulation factor Xa by antithrombin are accompanied by a ΔH of -20 to -31 kcal mol^{-1}. We observe no heat release upon proteolytic cleavage of inactive serpins or following inhibition of serine proteinases by canonical inhibitors or upon acylation of chymotrypsin by *N-trans*-cinnamoylimidazole. We suggest that part of the large enthalpy change that occurs during the structural transition of serpins is used to stabilize the proteinase in its inactive state.

The enzymatic activity of serine proteinases is regulated by two types of protein proteinase inhibitors: the so-called "canonical" inhibitors and the serpins. The former are relatively small proteins (29−190 amino acid residues) that belong to numerous structural families (*1*). The latter are larger proteins (400−450 residues) that form a single family with highly conserved secondary structural elements (nine α-helices and three β-sheets) (*2*).

Canonical inhibitors and serpins have quite different reaction mechanisms. The former are tight-binding reversible inhibitors with a short and rigid reactive site loop that forms a "lock and key" complex with the substrate binding site of the proteinase. This Michaelis-type complex is stabilized by a large number of noncovalent bonds which account for the high enzyme−inhibitor binding energy (*1*). In contrast, serpins are irreversible enzyme inhibitors with a long and flexible reactive site loop formed of ∼20 amino acid residues (from P_{15} to P'_6). They also form a Michaelis-type complex with their cognate proteinases, but this complex represents the initial but not the final inhibitory complex. The final complex is irreversible and SDS-stable as a result of the formation of a nonhydrolyzable acyl−enzyme bond between the serine residue of the catalytic site of the proteinase and the carbonyl group of the P_1 residue of the inhibitor. It is commonly believed that the serpin's $P_1-P'_1$ bond is cleaved within the initial Michaelis-type complex and that this cleavage triggers a conformational change that translocates the proteinase and stabilizes the acyl−enzyme bond (*3*, *4*). Recent data, however, suggest a different inhibition mechanism in which acylation of the proteinase does not take place within the Michaelis-type complex but within a translocated complex. In the latter mechanism, translocation of the enzyme is triggered by the formation of the Michaelis-type complex and not by cleavage of the serpin's $P_1-P'_1$ bond (*5*).

A recent crystallographic study shows that in the final inhibitory complex the $P_{15}-P_1$ part of the reactive site loop of the serpin inserts into β-sheet A to form the central strand of this sheet. On the other hand, the proteinase located on the opposite site of the inhibitor forms a stable acyl bond between the serine residue of its catalytic site and the P_1 residue of the serpin (*6*). Interestingly, proteolytic cleavage of the reactive site loops of serpins without formation of stable inhibitory complexes results in loop−sheet interactions similar to that described above (*7*−*9*). Also, a spontaneous P_4-P_{16} insertion into the middle of β-sheet A takes place when the serpin plasminogen activator inhibitor I undergoes an active → latent conversion (*10*). This profound conformational change leads to a large increase in conformational stability as shown by an increased resistance against unfolding by guanidinium hydrochloride (*11*) and an increased thermal stability (*12*, *13*).

Thus, active serpins with an exposed reactive site loop are metastable proteins which isomerize into a more stable but inactive state with a partially buried reactive site loop. Although the energy difference between the loop-inserted and the loop-exposed isomers is thought to be very large, it has never been quantitated. In this paper, we attempt to fill this gap by measuring the amount of heat released following reaction of serpins with proteinases that form or do not form complexes with them.

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MATERIALS AND METHODS

HLE[1] and PPE were purified according to published procedures (*14*, *15*). Bovine pancreatic chymotrypsin, porcine pancreatic trypsin, and PsE came from Worthington, Sigma, and Nagase, respectively. V8 proteinase from *Staphylococcus aureus*, papain, and coagulation factor Xa were from Boehringer Mannheim, Sigma, and Enzyme Research Laboratories, respectively. Recombinant α_1PI, eglin c, and mucus proteinase inhibitor were gifts from Novartis. Antithrombin was from Athens Research Technology. The antithrombin-binding pentasaccharide derived from heparin was kindly provided by M. Petitou (Sanofi, France). *N-trans*-Cinnamoylimidazole came from Sigma. The *p*-nitroanilide substrates came from Bachem. Enzymatic and calorimetric measurements were carried out in 50 mM Hepes and 150 mM NaCl (pH 7.4), a solution called the buffer.

Active Site Titrations. HLE and PPE were active site titrated with acetyl-Ala_2-Pro-AzaAla-*p*-nitrophenyl ester (*16*) (Enzyme System Products). Chymotrypsin and trypsin were titrated with *N-trans*-cinnamoylimidazole (*17*) (Sigma) and *p*-guanidinobenzoate (*18*) (Sigma), respectively. Titrated HLE was used to measure the active site titer of α_1PI (*19*), mucus proteinase inhibitor (*19*), and eglin c (*20*). Antithrombin was titrated as follows. Trypsin (66 nM) was incubated with increasing concentrations of inhibitor for 30 min at 25 °C in the buffer, and the residual enzymatic activity of the mixtures was measured at 410 nm with 1 mM benzoyl-L-arginine *p*-nitroanilide. The concentration of active antithrombin was deduced form the linear inhibition curve. The molarities of the other enzyme solutions were calculated using published molecular weights. Factor Xa was dialyzed against the buffer before use.

Preparation of a Binary α_1PI−Peptide Complex. The binary complex between α_1PI and Ac-Thr-Glu-Ala-Ala-Gly-Ala-Met-Phe-Leu-Glu-Ala-Ile-Val-Met (synthesized for us by Neosystem) was formed by reacting 175 μM α_1PI with a 15-fold molar excess of peptide under the conditions described by Schultze et al. (*21*). Excess peptide was removed by dialysis against the buffer. Full peptide incorporation was checked by (i) nondenaturating polyacrylamide gel electrophoresis which separates free and peptide-bound α_1PI (*22*) and (ii) the lack of inhibition of PPE (*21*). The latter was assessed by reacting constant concentrations of PPE (47 nM) with increasing concentrations of the binary α_1PI−peptide complex (from 29 nM to 1.4 μM) for 30 min at 25 °C and measuring the residual elastase activity with 0.5 mM succinyl-$(Ala)_3$-*p*-nitroanilide. When α_1PI was treated in the same way without peptide, it did not lose its antielastase activity.

Microcalorimetry. Microcalorimetric measurements were carried out using either a flow-mix measuring vessel or a microtitration ampule. Both devices were built in a heat conduction isothermal microcalorimeter (model 2277 thermal activity monitor, Thermometric). In this instrument, the heat produced (or absorbed) by the reacting sample is transferred to (or from) the surrounding heat sink. There is no feedback input of power, the temperature of the jacket being maintained to a very precise value by the water bath. A sensor for this heat flow (Peltier effect plates) generates an electrical potential, the output signal, which is directly proportional to the temperature difference between the reaction vessel and the heat sink. Data acquisition and processing were performed using Digitam software from Thermometric.

Most experiments were carried out by bringing the enzyme and the inhibitor together inside the calorimeter using the flow-mixing vessel permanently mounted in the apparatus. Separate solutions of enzyme and inhibitor were continuously injected into the calorimeter with identical flow rates using a two-channel peristaltic pump. These solutions passed through a double circuit which ensured their temperature equilibration before reaching the mixing cell and ultimately the 0.6 mL golden flow vessel (internal diameter of 1.0 mm) located in the heat-sensing part of the instrument. The thermal power (*P*), that is, the rate of heat production, was monitored until it reached a stable value (15−20 min). Data acquisition was carried out using the most sensitive scale, namely, 3 μW. Under the experimental conditions described here, the background noise was on the order of 50 nW. ΔH, the calorimetrically measured enthalpy change, was obtained from *P* using (*23*)

$$\Delta H = P/(f[\mathrm{I}]_o) \qquad (1)$$

where $[I]_o$ is the final inhibitor concentration and *f* the flow rate of the reaction mixtures. All concentrations given in the text and the figure legends are concentrations after mixing.

Control experiments in which the proteinase or the inhibitor was mixed with buffer showed that the thermal effect due to dilution was negligible. For each enzyme−inhibitor pair, the flow rate was adjusted to ensure full achievement of the reaction in the heat-sensitive zone of the apparatus. When required, the reaction media leaving the flow-mix vessel were analyzed by SDS−polyacrylamide gel electrophoresis to confirm the completeness of the reaction. The flow rate was assumed to be satisfactory when it was linearly related to *P*, that is, when it yielded constant ΔH values (see eq 1). It ranged from 5.0 to 7.5 $\times$ 10^{-3} mL s^{-1} depending on the enzyme−inhibitor pair.

A prerequisite for using the flow-mix device is that the enzyme−inhibitor reactions be sufficiently fast to be completed in less time than is required for the mixed solutions to pass through the heat-sensitive part of the calorimeter (80−90 s). This was not the case for the following systems: papain and α_1PI, V8 proteinase and α_1PI, HLE and antithrombin, and chymotrypsin and *N-trans*-cinnamoylimidazole. For these systems, the calorimetric measurements were carried out using a stainless steel microtitration ampule (Thermometric) inserted in the isothermal microcalorimeter described above. A small aliquot of enzyme was injected into 1 mL of the buffered inhibitor solution contained in the ampule. Stirring at 50 rpm was maintained during injection and data acquisition. This procedure is 5−10-fold less sensitive than flow-mix calorimetry and requires injecting very concentrated enzyme solutions. It allows, however, reaction heat variations to be recorded whatever the reaction rate of the partners.

RESULTS

Serpins may act as proteinase substrates or proteinase inhibitors. Both these reactions involve proteolysis of the

[1] Abbreviations: α_1PI, α_1-proteinase inhibitor (α_1-antitrypsin); PPE, porcine pancreatic elastase; HLE, human leukocyte elastase; PsE, *Pseudomonas aeruginosa* elastase.

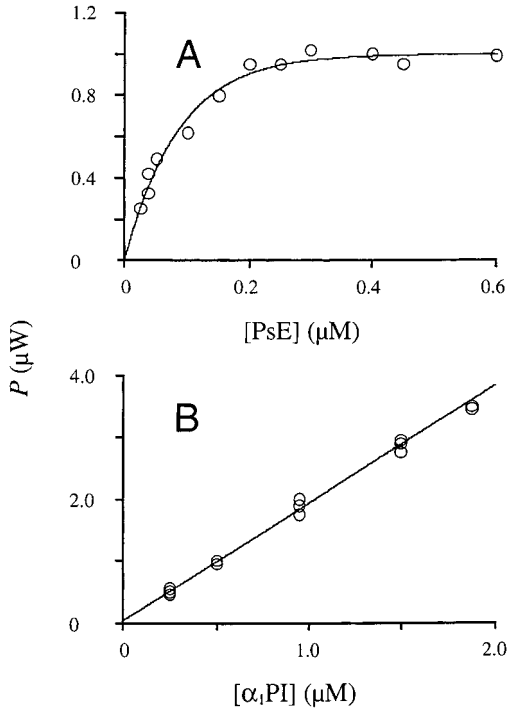

FIGURE 1: Thermal power (*P*) generated by the reaction of α_1PI with PsE at pH 7.4 and 25 °C in the flow-mix calorimeter. (A) Reaction of increasing concentrations of PsE with constant concentrations of α_1PI (0.5 μM). (B) Reaction of constant concentrations of PsE (0.3 μM) with increasing concentrations of α_1PI.

reactive center loop and insertion of the newly formed C-terminal peptide into β-sheet A in forming the central strand of this sheet (*6*, *7*). This dramatic structural alteration renders the reaction of serpins with proteinases thermodynamically complete. Hence, ΔG values for serpin reactions cannot be determined from the calorimetric data. The only thermodynamic parameter that can be calculated is ΔH.

Heat Production following Cleavage of α_1PI by PsE, Papain, and V8 Proteinase. PsE inactivates the serpin α_1PI by a limited proteolytic cleavage of the $Pro_{357}-Met_{358}$ (P_1-P_2) bond of the inhibitor (*24*). When constant concentrations of α_1PI were reacted with increasing concentrations of PsE in the flow-mix apparatus, the thermal power *P* progressively increased and reached a plateau (P_{sat}) corresponding to the cleavage of all the α_1PI molecules that were present (Figure 1A). Full cleavage of α_1PI at its active center was checked by SDS−polyacrylamide gel electrophoresis (*24*). The average enthalpy change, ΔH, was -63 kcal mol^{-1}.

Figure 1B reports the thermal power of mixtures formed of constant concentrations of PsE and increasing concentrations of α_1PI. In all of these mixtures, the totality of α_1PI was cleaved. There was no heat production in the absence of α_1PI, indicating that the calorimetric signal is very specific. On the other hand, *P* is strictly proportional to the concentration of α_1PI and therefore represents a quantitative measurement of the extent of cleavage of α_1PI. The enthalpy change was calculated for each experiment whose results are depicted in Figure 1B, and its average value was found to be -62 kcal mol^{-1}, which confirms the above figure.

ΔH for the α_1PI−PsE system has also been measured between 12 and 48 °C. Figure 2 shows that it decreases with temperature, which corresponds to a heat capacity change ΔC_p of -566 cal K^{-1} mol^{-1}.

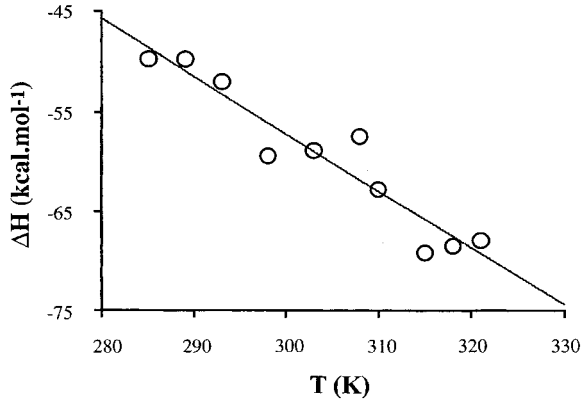

FIGURE 2: Effect of temperature on the enthalpy of the reaction of 0.55 μM α_1PI with 0.1−0.38 μM PsE at pH 7.4 as measured by flow-mix calorimetry. Preliminary experiments showed that the cleavage of α_1PI by PsE was complete within the heat-sensitive zone of the apparatus and that no heat inactivation of α_1PI and PsE occurred at the higher temperatures. Linear regression analysis yielded a slope (ΔC_p) of -566 ± 90 cal K^{-1} mol^{-1}.

Table 1: Enthalpy of the Reaction for Serpin−Proteinase Interactions at pH 7.4 and 25 °C[a]

serpin	proteinase	calorimetric method	ΔH (kcal mol^{-1}) (average values from several experiments ± SD)
α_1PI	PsE	flow-mix	-63.0 ± 4.6[b]
α_1PI	V8 proteinase	titration	-52.5 ± 5.0[c]
α_1PI	papain	titration	-52.7 ± 3.0[d]
α_1PI	PPE	flow-mix	-28.0 ± 1.1[e]
α_1PI	HLE	flow-mix	-26.0 ± 2.2[e]
α_1PI	Chy	flow-mix	-23.4 ± 1.5[f]
AT	HLE	titration	-63.4 ± 8.5[g]
AT-hp	HLE	flow-mix	-62.7 ± 8.4[g]
AT	Try	flow-mix	-20.0 ± 2.0[h]
AT-hp	Try	flow-mix	-22.2 ± 2.0[h]
AT-hp	factor Xa	titration	-31.0 ± 4.5[i]

[a] AT, antithrombin; hp, heparin pentasaccharide; Chy, bovine pancreatic chymotrypsin; Try, porcine pancreatic trypsin; SD, standard deviation. [b] See Figure 1. [c] With 28 μM α_1PI and 0.66 μM V8 proteinase. [d] With 28 μM α_1PI and 0.1 μM papain. [e] See Figure 3. [f] See Figure 4. [g] With 17 μM AT and 0.7 μM HLE with or without 39 μM hp. [h] With 2.1 μM AT and 3.3 μM Try with or without 39 μM hp. [i] With 0.85 μM AT, 2.2 μM factor Xa, and 39 μM hp (hp was mixed with both AT and HLE, Try, or factor Xa to avoid a thermal effect due to its dilution).

We also used microtitration calorimetry to follow the substrate-like cleavage of α_1PI by papain and V8 proteinase which hydrolyze the reactive site loop at the P_6-P_7 and P_4-P_5 bonds, respectively (*25*, *26*). The enthalpy changes were found to be -52.7 and -52.5 kcal mol^{-1}, respectively (Table 1).

Heat Production following Inhibition of Serine Proteinases by α_1PI. α_1PI inhibits a number of serine proteinases, including PPE, HLE, and chymotrypsin (*27*). Figure 3A shows that the thermal power *P* increases linearly and reaches a maximum P_{sat} following addition of increasing concentrations of PPE to a constant concentration of α_1PI. The inset of Figure 3A depicts the results of a similar experiment with HLE. In both cases, P_{sat} is reached when 1 mol of enzyme has reacted with 1 mol of inhibitor. The enthalpy change ΔH was calculated using the average values of P_{sat} and was found to be -27 kcal mol^{-1} for the α_1PI−PPE interaction and -25.7 kcal mol^{-1} for the α_1PI−HLE interaction.

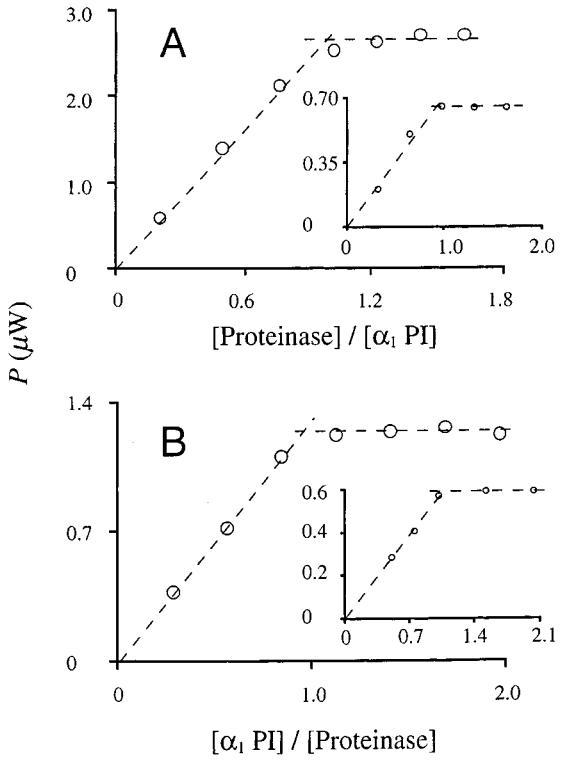

FIGURE 3: Thermal power (*P*) generated by the reaction of α_1PI with PPE and HLE at pH 7.4 and 25 °C in the flow-mix calorimeter. (A) Reaction of increasing concentrations of PPE (main figure) or HLE (inset) with constant concentrations of α_1PI (3.0 and 0.95 μM, respectively). (B) Reaction of constant concentrations of PPE (1.5 μM, main figure) and HLE (0.8 μM, inset) with increasing concentrations of α_1PI.

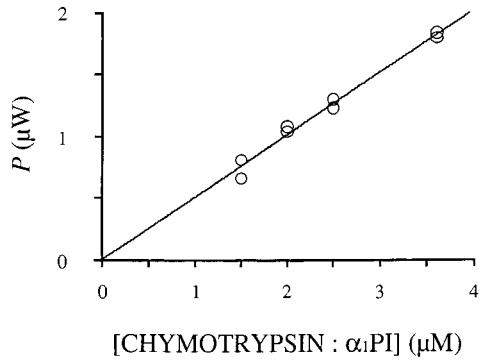

FIGURE 4: Thermal power (*P*) generated by the reaction of equimolar concentrations of bovine pancreatic chymotrypsin and α_1PI at pH 7.4 and 25 °C in the flow-mix calorimeter.

These data could be confirmed by the reverse titration experiment whose results are shown in Figure 3B; again, P_{sat} became constant when the enzyme and inhibitor concentrations were equal, and ΔH was close to that found above: −29 and −26.8 kcal mol^{-1} for the reaction of α_1PI with PPE and HLE, respectively.

Figure 4 shows that the thermal power generated by the reaction of equimolar concentrations of bovine pancreatic chymotrypsin and α_1PI is strictly proportional to the concentration of the enzyme−inhibitor complex. The average value of ΔH was −23.4 kcal mol^{-1}.

Heat Production following Reaction of Antithrombin with Trypsin, Factor Xa, and HLE. Antithrombin inhibits thrombin, factor Xa, and other trypsin-like enzymes such as pancreatic trypsin (*28*). HLE inactivates this serpin by proteolytic cleavage at its reactive center loop (*29*). This nonproductive cleavage was accompanied by an enthalpy change of −63.4 kcal mol^{-1}, whereas complex formation between antithrombin and porcine pancreatic trypsin was characterized by a ΔH of −20 kcal mol^{-1} (Table 1). Prior reaction of antithrombin with the rate-enhancing heparin pentasaccharide (*30*) did not significantly change the heat generated by the action of HLE or trypsin. On the other hand, inhibition of factor Xa by pentasaccharide-bound antithrombin was accompanied by a ΔH of −31 kcal mol^{-1}.

Lack of Heat Production following Reaction of Proteinases with Inactive Serpins, Canonical Inhibitors, and an Acylating Agent. We have used two inactive serpins: (i) chicken ovalbumin which is unable to insert the P_1-P_{14} peptide into β-sheet A following cleavage by serine proteinases and (ii) the in vitro-prepared binary α_1PI−(P_1-P_{14}) complex in which the synthetic P_1-P_{14} peptide inserts into β-sheet A and thus prevents the insertion of the P_1-P_{14} peptide of the inhibitor (*21*). Proteinases cleave this derivative at the reactive center loop but are not inhibited by it (*21*). No heat was released upon reacting 4.5 or 9.0 μM ovalbumin with 0.85 μM PPE. On the other hand, reaction of 2.9, 5.8, and 8.7 μM binary α_1PI−(P_1-P_{14}) complex with 0.53 μM PsE or reaction of 1.6 or 3.2 μM binary complex with 1.9 μM PPE gave no significant heat release. Using polyacrylamide gel electrophoresis, we have checked that ovalbumin and the α_1PI−(P_1-P_{14}) complex are cleaved under the conditions described above.

The mucus proteinase inhibitor and eglin c are canonical inhibitors that form very tight reversible complexes with proteinases (*1*). To test the heat generation potential of these inhibitors, we used them at concentrations close to those of the heat-generating serpins. No heat production was observed upon reacting 6.6 μM mucus proteinase inhibitor with 6.6 μM HLE or 7.7 μM eglin c with 7.7 μM chymotrypsin in the flow-mix calorimeter. Under these concentration conditions, there is full enzyme−inhibitor association.

We also used microtitration calorimetry to follow the acylation of the catalytic serine residue of chymotrypsin by the active site titrant *N-trans*-cinnamoylimidazole (*17*). No heat release was observed when 46 μM chymotrypsin was reacted with 12 μM titrant.

DISCUSSION

Serpins behave as either proteinase substrates or proteinase inhibitors. We have shown that each of these reactions involves an important enthalpy change. When active serpins behave as proteinase substrates, they are inactivated by proteolysis at their reactive site loop and the newly formed C-terminal peptide deeply inserts into the middle of five-stranded β-sheet A to form a new strand, called s4A (*7*−*9*). We believe it is this and other structural modifications but not the proteolytic event that cause the observed heat production because there is no heat release upon reaction of proteinases with ovalbumin, or with the binary α_1PI−(P_1-P_{14}) complex, both of which are proteolyzed at their "reactive site" but are unable to undergo further structural changes (*13, 21*).

The substrate-like cleavage of α_1PI by PsE, V8 proteinase, and papain takes place at the P_1-P_2, P_4-P_5, and P_6-P_7 bonds, respectively. The peptides that insert into β-sheet A

are, therefore, significantly shorter in papain or V8 proteinase-hydrolyzed α_1PI than in the PsE-cleaved inhibitor. Yet, ΔH is only 13% lower for the papain- or V8 proteinase-catalyzed cleavages than for the PsE−α_1PI interaction, suggesting either that β-sheet interaction of the distal amino acid residues releases much less heat than that of the proximal ones or that part of the observed heat release arises from the gross serpin body conformational change. The latter possibility is in agreement with recent findings by Im et al. (*32*) and Seo et al. (*33*), suggesting that metastability of native α_1PI results from constraints due to unfavorable interactions scattered in the whole core of the protein. Numerous structural changes accompanying the transition of α_1PI have also been described by Whisstock et al. (*34*), who analyzed the relative movements of the rigid subunits composing the core of α_1PI. It is thus possible that the heat effect observed during the substrate reaction of α_1PI with proteinase is due not only to the reactive site loop insertion but also to a series of structural events, a sequence of which is proposed by Whisstock et al. (*34*).

Antithrombin and α_1PI have sequences that are only 28% identical (*9*), and their reactive site loops are differently exposed to solvent (*35*, *36*). They also differ in heparin binding. The heparin pentasaccharide, the minimum fragment of heparin able to bind to antithrombin, significantly exposes the reactive site loop of antithrombin to solvent (*36*, *37*). Despite their structural differences, active α_1PI, antithrombin, and antithrombin-pentasaccharide release very similar amounts of energy upon proteolysis (Table 1), in accord with the fact that they undergo an identical conformational rearrangement.

The proteolysis of α_1PI by PsE is characterized by a large and negative change in heat capacity ($\Delta C_p = -566$ cal K^{-1} mol^{-1}). Large and negative ΔC_p values may be observed when solvent-exposed hydrophobic residues become buried in the core of proteins and/or when the protein structure is tightened (*38*−*40*). Thus, the ΔC_p observed with α_1PI is consistent both with the insertion into β-sheet A of strand s4A whose amino acid residues are mostly hydrophobic (*41*) and with the rearrangement of the rest of the molecule of α_1PI (*32*−*34*).

When serpins behave as proteinase inhibitors, they first bind proteinases to form a Michaelis-type complex which is subsequently translocated (*42*). The final complex is a nonhydrolyzable acyl−enzyme intermediate linking the serine residue of the proteinase with the P_1 residue of the serpin. The three-dimensional structure of this final complex shows that the reactive site loop of the serpin inserts into β-sheet A as it does during the substrate-like reaction (*6*). We have found that the inhibition of PPE, HLE, and chymotrypsin by α_1PI and the inhibition of trypsin and coagulation factor Xa by antithrombin are accompanied by a significant enthalpy change. Moreover, the calorimetric titration of PPE and HLE with α_1PI resembles the enzymatic titrations; the titration curves are linear and level off for a mole to mole interaction (Figure 3). This strongly suggests that the observed enthalpy change is due to the formation of the stable α_1PI−proteinase complexes. The enthalpy changes resulting from the formation of the six stable serpin−proteinase complexes do not differ greatly from each other (see Table 1), suggesting that the proteinase and serpin specificity and their rate of association (*27*) do not greatly influence the change in enthalpy. Also, the lack of heat release observed upon reacting proteinases with the canonical inhibitors eglin c or mucus proteinase inhibitor, whose binding energy arises from the formation of a tight Michaelis-type complex and not from a conformational change of the inhibitor, strongly suggests that the heat released by serpin reactions cannot be ascribed to the formation of the initial Michaelis-type complex. On the other hand, the enthalpy change is probably not due to the formation of the acyl bond between the serine residue of the proteinase and the P_1 residue of the inhibitor since acylation of the active center serine residue of chymotrypsin by the acylating agent *N-trans*-cinnamoylimidazole does not result in heat production.

Serpins release significantly less heat when they act as proteinase inhibitors ($\Delta H = -25$ kcal mol^{-1} on average) than when they behave as proteinase substrates ($\Delta H = -59$ kcal mol^{-1} on average). Since both inhibitor and substrate reactions are characterized by identical conformational alterations (*6*), one may suggest that part of the loop insertion energy may be used to stabilize the serpin−proteinase complex by distorting the conformation of the proteinase (*6*) and distorting the enzyme's catalytic site to prevent hydrolysis of the acyl−enzyme intermediate (*43*). In this context, the observation that the enthalpy change resulting from the inhibition of factor Xa by antithrombin is somewhat higher than that corresponding to the binding of trypsin to antithrombin (see Table 1) suggests that the stabilization of the antithrombin−factor Xa complex has somewhat fewer enthalpic requirements than that of the antithrombin−trypsin complex.

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