Introduction of a Mutation in the Shutter Region of Antithrombin (Phe77 → Leu) Increases Affinity for Heparin and Decreases Thermal Stability†

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ABSTRACT: The shutter region of serpins consists of a number of highly conserved residues that are critical for both stability and function. Several variants of antithrombin with substitutions in this region are unstable and predispose the carrier to thrombosis. Although most mutations in the shutter region investigated to date are deleterious with respect to serpin stability and function, the substitution of Phe51 by Leu in α_1-antitrypsin results in enhanced stability. Here, we have investigated the effects of introducing an analogous mutation into antithrombin (Phe 77 to Leu). The mutation did not affect the kinetics of interaction with proteases. Strikingly, however, the thermostability of the protein was markedly decreased, with the serpin displaying a 13 °C decrease in melting temperature as compared to wild-type recombinant antithrombin. Further studies revealed that in contrast to wild-type antithrombin, the mutant adopted the latent (inactive) conformation upon mild heating. Previous studies on shutter region mutations that destabilize antithrombin revealed that such variants possess enhanced affinity for both heparin pentasaccharide and full-length heparin. The N135A/F77L mutant had unchanged affinity for heparin pentasaccharide, but the affinity for full-length heparin was increased. We suggest that the Phe77Leu mutation causes conformational changes around the top of the D-helix in antithrombin, in particular, to the arginine 132 and 133 residues that may mediate additional antithrombin/heparin interactions. This paper also demonstrates that there are major differences between the shutter regions of antithrombin and α_1-antitrypsin since a stabilizing mutation in antitrypsin has the converse effect in antithrombin.

Antithrombin is a plasma serine proteinase inhibitor (serpin), which plays a major role in the regulation of serine proteases involved in the coagulation cascade (*1*). Inhibition of thrombin and factor Xa by antithrombin proceeds at a slow rate until the serpin binds to heparin, which acts via a specific pentasaccharide sequence (*2*) to induce a conformational change in antithrombin, yielding a more potent inhibitor. The induction of the conformational change, which particularly accelerates the inhibition of factor Xa (*3*), culminates in the expulsion of the partially inserted reactive center loop (RCL) from the A β-sheet to an exposed conformation more suitable for interaction with the target proteases (*4*). Higher molecular weight heparins containing the pentasaccharide sequence (denoted high affinity heparin; HAH) appear to have an action further to that of the pentasaccharide, postulated to be a bridging effect that acts to increase the rate at which the protease and serpin associate (*5*).

Previous studies on both natural and engineered variants have revealed that the shutter region is important for the stability and function of serpins (*6*). Many mutations in the shutter region are deleterious to both serpin stability and function, for example, two natural antithrombin variants, wibble (T85M) and wobble (T85K), are conformationally labile and possess enhanced affinity for heparin pentasaccharide and HAH (*7*). These data suggest that the shutter region is important for the transmission of a conformational change throughout the molecule from the heparin binding site to the RCL (*8*).

In this study, we were interested in further investigating the role of the shutter region in the structure and function of antithrombin and its interaction with heparin. Certain shutter region mutations in serpins have been shown to result in enhanced stability, in particular, the Phe51Leu variant of

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antitrypsin (*9*). In this study, we introduced the analogous mutation into antithrombin at position 77 (F77L) (equivalent to position 51 in antitrypsin). In contrast to the effect of this substitution in antitrypsin, the F77L variant of antithrombin displayed decreased stability and an increased susceptibility to adopt the latent form of antithrombin. It also had an unchanged affinity for heparin pentasaccharide but a higher affinity for longer heparin species.

EXPERIMENTAL PROCEDURES

Materials. Heparin pentasaccharide was a gift from Maurice Petitou, Sanofi Research, France. High affinity heparin (HAH) was prepared as described previously (*10*). Factor Xa was purchased from Boehringer-Mannheim. The BaculoGold kit was supplied by Pharmingen, San Diego, CA.

Production and Isolation of Variant Forms of Antithrombin. Site-directed mutagenesis of the antithrombin human cDNA was carried out using a two stage PCR system (*11*). The whole sequence was checked by DNA sequencing. Insect cell expression of N135A and N135A/F77L antithrombin molecules was carried out as previously described (*12*). Purification of recombinant protein from cleared supernatant was carried out using a modification of the method of McKay (*13*) in which active fractions from heparin-Sepharose were further purified on a Superdex 75 column.

Kinetic and Binding Assays. Kinetic experiments were carried out in a 20 mM NaH_2PO_4, 0.1% (w/v) PEG 8000, 0.1 mM EDTA, pH 7.4 buffer with the addition of 0.1, 0.25, 0.35, 0.45, or 0.55 M NaCl to give ionic strengths of 0.15, 0.3, 0.4, 0.5, or 0.6 I, respectively. Stoichiometry of inhibition and second-order rate constants were measured against thrombin and factor Xa as previously described (*14*). Equilibrium dissociation constants for pentasaccharide and HAH were determined by monitoring the intrinsic fluorescence enhancement during polysaccharide titrations as previously described (*14*).

The contribution of nonionic and ionic bonds to the binding of heparin and antithrombin was calculated by plotting the logarithm_{10} (log) of the ionic strength versus the log of the equilibrium binding constant (K_d) measured at this ionic strength (*3*). Linear regression analysis of this line is used to obtain the number of ionic interactions from the slope, when divided by the heparin charge density parameter (0.8), and also the number of nonionic interactions, which is represented by the ordinate intercept.

Thermal Stability of the Antithrombin Variants. Changes in the protein secondary structure were monitored by continuous measurement of the changes in the CD signal at 222 nm using a Jasco J-810 spectrapolarimeter. The protein samples (0.1 mg/mL) were incubated in 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, 0.1% (w/v) PEG 6000, pH 7.4, 0.15 I. The rate of temperature change within the cuvette was at 1 °C/min, and the temperature ranged from 25 to 90 °C. The thermal melt data are an average from three separate experiments. Data were fitted to the curve to yield a thermal melt temperature (T_m) as described by Huntington et al. (*15*).

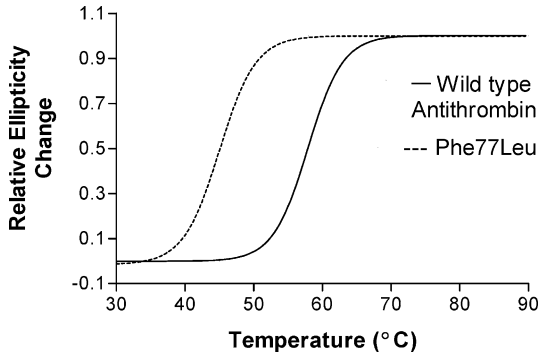

FIGURE 1: Thermal melting profiles of N135A (wild type) and N135A/F77L recombinant antithrombin molecules as monitored by continuous measurement of the changes in the CD signal at 222 nm.

RESULTS

Production of Recombinant Antithrombin. Throughout this study, the β form of antithrombin (N135A) was used as the wild-type background. The N135A mutation has been shown to generate a homogeneous form of antithrombin in the *Baculovirus* expression system (*12*). Sequencing of both the N135A and the N135A/F77L clones demonstrated the presence of mutagenic nucleotides and absence of other mutations in the DNA. Purification of both N135A and N135A/F77L recombinant proteins on heparin-Sepharose and Superdex-75 yielded pure proteins as judged by nondenaturing PAGE and SDS−PAGE (results not shown).

Kinetics of Inhibition. Both recombinant antithrombin molecules were shown to have a stoichiometry of inhibition for thrombin and factor Xa of one and exhibited similar second-order rate constants of association (k_{ass}) with factor Xa (k_{ass} values were $4.2 \pm 0.33 \times 10^3$ M^{-1} s^{-1} and $4.94 \pm 0.15 \times 10^5$ M^{-1} s^{-1} in the absence and presence of pentasaccharide, respectively, for N135A and $4.34 \pm 0.24 \times 10^3$ M^{-1} s^{-1} and $4.49 \pm 0.03 \times 10^5$ M^{-1} s^{-1} for N135A/F77L). Similarly, the introduction of the mutation also had no effect on k_{ass} between thrombin and the variants ($1.1 \pm 0.05 \times 10^4$ M^{-1} s^{-1} for N135A and $1.1 \pm 0.18 \times 10^4$ M^{-1} s^{-1} for N135A/F77L in the absence of pentasaccharide).

Stability Studies. The stability of the recombinant antithrombin molecules was assessed in comparison to plasma antithrombin, by analysis of thermal melting curves using far-UV circular dichroism (CD) spectroscopy (*15*). The plasma and recombinant N135A forms of antithrombin had similar midpoints of thermal denaturation (T_m) [59 °C ± 1 for plasma antithrombin and 58 °C ± 2 for N135A], but the N135A/F77L mutant was considerably decreased in its stability toward heat, with its T_m decreased by 13−45 °C ± 2 (Figure 1). Exposing serpins to heat commonly leads to one of two outcomes, the formation of the so-called latent form, in which the RCL of the serpin inserts into its own A β-sheet, thus inactivating it (*16*), or polymers of serpins, in which the RCL of one serpin inserts into the A or C β-sheet of another serpin molecule, thus propagating the formation of a polymeric form of the molecule (*17*−*19*). We incubated N135A and N135A/F77L recombinant antithrombin at 40 °C for 1 h, following which the proteins were chromatographed on a Superdex 75 gel filtration, and the inhibitory activity of the eluted fractions was measured. No polymer peak (expected to elute at the void volume) was detected

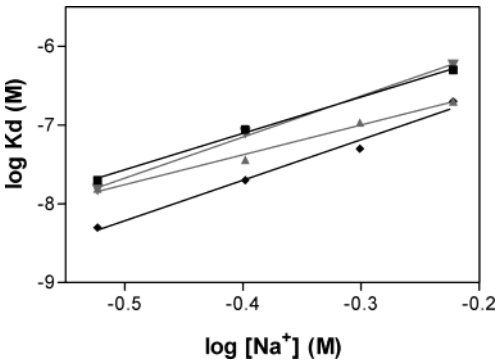

FIGURE 2: Ionic strength dependence of the binding of high affinity heparin species to recombinant antithrombin. Log_{10} of the observed equilibrium binding constants of high affinity heparin with N135A (▲) and N135A/F77L (◇) and heparin pentasaccharide with N135A (■) and N135A/F77L (▼) were plotted against the log of the ionic strength at which the equilibrium binding constant was obtained.

for either protein, and each eluted at the position expected for monomeric antithrombin. Upon testing for activity, N135A antithrombin was found to be fully active, while N135A/F77L antithrombin was found to be completely inactive. This led us to conclude that the heating of the N135A/F77L variant caused it to adopt the latent conformation, while N135A antithrombin was stable to the conditions used in the experiment.

Interaction with Heparin. The conformational change induced in antithrombin upon pentasaccharide binding may be monitored through characteristic changes in intrinsic tryptophan fluorescence (*3*). The fluorescence emission spectra of N135A and N135A/F77L in the presence and absence of pentasaccharide showed that the percentage change in emission at the peak of the spectra (340 nm) upon the addition of pentasaccharide was 32.1 ± 2.5% for N135A and 30.1 ± 1.6% for N135A/F77L (data not shown). This indicates that the extent of the pentasaccharide induced conformational change was not significantly altered by the F77L mutation.

The affinity of N135A and N135A/F77L for heparin pentasaccharide and high affinity heparin were measured using equilibrium binding titrations obtained by monitoring the changes in intrinsic fluorescence of the protein on the addition of heparin species. Analysis at ionic strengths of 0.3, 0.4, and 0.5 revealed that the affinity of the N135A/F77L mutant for heparin pentasaccharide was not significantly changed as compared to that of recombinant β antithrombin (N135A) [Figure 2]. The equilibrium dissociation constants (K_d) of the two antithrombin forms for HAH were also determined at four ionic strengths, revealing that the K_d at 0.3 I was 3-fold lower for the N135A/F77L mutant than for N135A antithrombin, at 0.4 I it was 1.7-fold lower, and at 0.5 I it was 2-fold lower (Figure 2). At an ionic strength of 0.6, the K_d was approximately the same for both recombinant proteins. Hence, the binding of high affinity heparin to the N135A/F77L mutant was more favorable at low ionic strengths, but as the ionic strength increased the effect was negated, indicating a contribution from the ionic bonds between the high affinity heparin and the recombinant antithrombin.

The contribution that nonionic and ionic bonds made to the interaction between the recombinant proteins and high affinity heparin was calculated from the equilibrium dissociation constants, using a double logarithmic plot of ionic strength versus K_d as previously described (Figure 2). Analysis of these data gave slopes of 3.82 ± 0.27 and 5.24 ± 0.34 for the N135A and N135A/F77L forms, respectively, which indicates that the contribution from ionic bonds is 4.8 for N135A and 6.6 for N135A/F77L binding to high affinity heparin. The nonionic interactions were quantitated as 5.85 ± 0.10 for N135A and 5.56 ± 0.13 for N135A/F77L. These results indicate that the equivalent of two extra sodium ions is liberated during the interaction between HAH and N135A/F77L, while the number of nonionic interactions contributing to the binding remains essentially the same.

DISCUSSION

In this study, a mutation was introduced into the shutter region of antithrombin at an equivalent position to that which had a stabilizing effect on another serpin, α_1-antitrypsin (*9*). Initially, Kwon et al. (*20*) identified the Phe 51 residue in α_1-antitrypsin as being important for thermal stability of α_1-antitrypsin by using a random mutagenesis approach, which yielded a Phe51Cys mutant with enhanced stability. Further mutagenesis studies showed that increasing the size of the side chain at position 51 decreased stability, while decreasing size stabilized the protein, establishing important rules for the overall functioning of the shutter region, which were subsequently borne out by analysis of a large amount of data from natural serpin variants (*5*). This helped to define the shutter region of the serpins as being vital in the control of stability of serpins, mostly because of the importance of the region in controlling the mobility of the A β-sheet strands, which in turn is pivotal to serpin function and stability.

Previous studies on natural variants of antithrombin with mutations in the shutter region (N187D, T85M, and T85K) (*7*, *21*) have revealed that the portion of the shutter that abuts the heparin binding helix (the D-helix) is both important for stability and important for the transmission of a conformational change from the heparin binding site to the RCL (*8*). Interestingly, all of these variants possessed enhanced heparin affinity, and it was reasoned that destabilizing mutations in antithrombin that result in spontaneous RCL insertion into the A β-sheet (i.e., conversion to the latent or polymeric state) would logically also possess an enhanced ability to undergo conformational rearrangement to the heparin activated, RCL expelled form (*8*). In this study, we were interested to establish whether the mutation (F77L) that we predicted would have the opposite effect (i.e., result in a variant possessing enhanced stability) would lower the affinity for heparin pentasaccharide and HAH.

The N135A/F77L form was shown to be similar to recombinant β-antithrombin in its behavior in a number of systems, having similar SI and k_{ass} with factor Xa (in the presence and absence of heparin) and thrombin and a similar conformational change upon binding heparin pentasaccharide.

To our surprise, the N135A/F77L form was dramatically destabilized in terms of its heat stability as compared to the recombinant β-form, forming the latent form readily at 40 °C. Thus, it seems likely that the environment surrounding Phe77 in antithrombin must be quite different from α_1-antitrypsin, where the effect of introducing a smaller side chain is to stabilize rather than destabilize the molecule.

To investigate the affinity of the N135A/F77L mutant for heparin pentasaccharide and HAH, the equilibrium binding

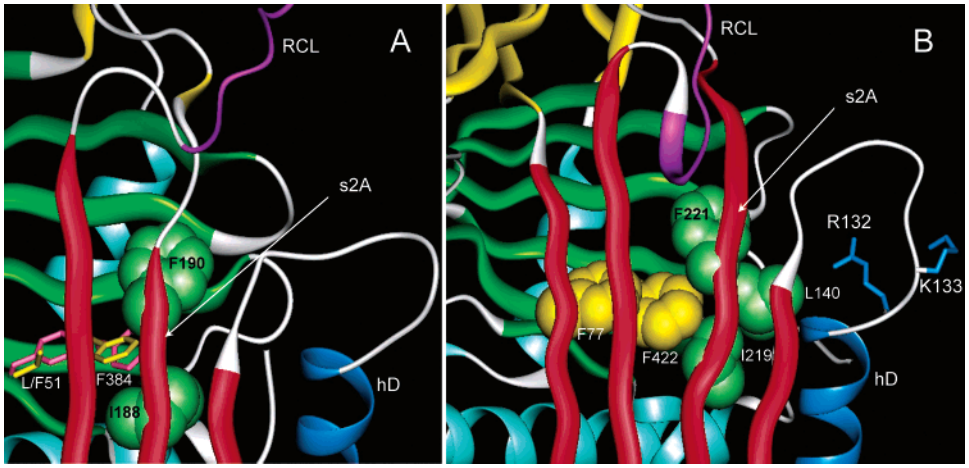

FIGURE 3: (A) Structure of wild-type (PDB 1QLP) α_1-antitrypsin with the position of F51 and F384 highlighted in pink. The positions of L51 and F384 in F51L antitrypsin (PDB 1PSI) are in yellow. I188 and F190 are in light green van der Waal spheres. The D-helix is in dark blue and the RCL in magenta. The A-sheet is in red ribbon, the B-sheet in green, and the C-sheet in yellow. Strand s2A of the A-sheet is indicated. (B) The shutter region of native, partially inserted antithrombin (PDB 2ANT). Coloring is as in panel A. F77 and F422 are represented by van der Waals spheres and are labeled. F221, I219, and L140 are labeled. R132 and K133 are in dark blue. Strand s2A of the A-sheet is indicated.

constants were determined at several ionic strengths. The equilibrium binding constants for the interaction of the N135A/F77L form with the specific heparin pentasaccharide were essentially the same as those for N135A, within experimental error. Thus, the N135A/F77L variant is unlikely to have affected the conformation of the primary heparin pentasaccharide binding residues. Furthermore, since the rate of conformational change induced by heparin pentasaccharide is unaffected, these data suggest a role for F77 distinct from the conformational pathway affected in the antithrombin variants T85M and T85K.

The F77L form was shown to have increased affinity for HAH. This was shown to be due to the equivalent of two more ionic bonds being formed when the N135A/F77L bound full-length heparin, in comparison to the N135A form. The structures of wild-type α_1-antitrypsin (*22*), F51L α_1-antitrypsin (*23*), native antithrombin (*24*), and pentasaccharide-bound antithrombin (*4*) were examined to elucidate possible reasons for the decreased thermal stability in the antithrombin mutant and the high affinity for long chain heparins.

A close examination of the packing around position F51 in the three-dimensional structures of wild type and the F51L variant of α_1-antitrypsin revealed that the F51 mutant in α_1-antitrypsin has only one major effect, which is a significant movement in the side chain of F384 (Figure 3A) (*22*, *23*). No other changes are seen, most likely because the movement in F384 does not result in disruption of the packing around the top of the A β-sheet. However, F384 is located in a critical position, in that it packs between F190 and I188, and these two conserved residues slide across the underlying B β-sheet during the conformational change to the cleaved (or inserted) state (*8*). Hence, the increase in stability of the F51 mutant can be explained by the adjustment in the side chain of F384 resulting in more favorable packing of F190 and I188, making the movement of these residues and consequent conformational changes, especially insertion of the α_1-antitrypsin RCL, less favorable. Therefore, why does the F77L mutation in antithrombin result in a decrease in stability? It is argued that since antithrombin exists in a partially inserted conformation, the stability of N135A/F77L antithrombin would not be enhanced in an analogous manner to α_1-antitrypsin since the RCL of antithrombin has already inserted to the P14 position, whereas in α_1-antitrypsin it is predicted that the F51L mutation would act to disfavor this initial insertion.

The partial insertion of the reactive center loop of antithrombin into the top of the A β-sheet results in the residues equivalent to F190 (F221) and I188 (I219) in α_1-antitrypsin sliding across the underlying B β-sheet to allow the insertion (see Figure 3B). As in α_1-antitrypsin, F221 and I219 pack against F422 (equivalent to F384 in α_1-antitrypsin). In antithrombin, F221 also packs against L140 on strand s2A of the A β-sheet, which in turn packs against the loop joining strand s2A to the top of the D-helix (see Figure 3B). It is suggested that the mutation of the Phe77 to a Leu residue would cause a change in conformation of F422 that in turn may cause a change in the conformation of F221. F221 is located directly underneath the partially inserted RCL, and we suggest that a change in conformation in this residue may promote further RCL insertion, thus explaining the overall decrease in stability of the molecule. In addition, a change in conformation of the side chain of F221 may also have a knock on effect to L140, in turn affecting the conformation of residues at the top of the D-helix. More specifically, the loop joining strand s2A to the D-helix contains two residues that have been shown to be important for binding full length heparin—Arg132 and Lys133 (*25*). In native antithrombin, this loop is thought to be flexible (*24*). The structure of native antithrombin bound to heparin pentasaccharide (*4*) has shown that this loop forms an extra two turns at the top of the D-helix, bringing Arg132 and Lys 133 in line with the pentasaccharide binding residues. Thus, the F77L mutation may change the conformation of the loop joining s2A to the D-helix and bring Arg132 and Lys133 into a more suitable conformation for initial binding to long chain heparin, resulting in the equivalent of an extra two ionic interactions in the initial binding of long chain heparin to N135A/F77L antithrombin.

Thus, the initial hypothesis that the F77L mutation in the shutter region of antithrombin would create a stabilized mutant that would be of interest to explore in terms of its interaction with heparin was proven incorrect. What was eventually obtained was a destabilized mutant that interestingly had higher affinity specifically for longer chain heparin. The results obtained in this study highlight the differences between antithrombin and other serpins, mainly because of the partially inserted RCL of this serpin and the changes this engenders in the shutter region of the molecule. The shutter region of serpins is vital in the control of the serpin mechanism and also for stability. The results of this study highlight that antithrombin is perhaps more precariously balanced than other serpins in terms of the balance between partial and full insertion of its RCL into the A β-sheet.

REFERENCES

1. Rosenberg, R. D. (1975) Actions and interactions of antithrombin and heparin, *New Engl. J. Med. 292*, 146−151.
2. Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B., and Gatti, G. (1983) Structure−activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity, *Biochem. Biophys. Res. Commun. 116*, 492−499.
3. Olson, S. T., Bjork, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin−proteinase reactions. Resolution of the antithrombin conformational change contribution to heparin rate enhancement, *J. Biol. Chem. 267*, 12528−12538.
4. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) The anticoagulant activation of antithrombin by heparin, *Proc. Natl. Acad. Sci. U.S.A. 94*, 14683−14688.
5. Olson, S. T., and Bjork, I. (1991) Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin−thrombin reaction, *J. Biol. Chem. 266*, 6353−6364.
6. Stein, P. E., and Carrell, R. W. (1995) What do dysfunctional serpins tell us about molecular mobility and disease? *Nat. Struct. Biol. 2*, 96−113.
7. Beauchamp, N. J., Pike, R. N., Daly, M., Butler, L., Makris, M., Dafforn, T. R., Zhou, A., Fitton, H. L., Preston, F. E., Peake, I. R., and Carrell, R. W. (1998) Antithrombins Wibble and Wobble (T85M/K): archetypal conformational diseases with in vivo latent-transition, thrombosis, and heparin activation, *Blood 92*, 2696−2706.
8. Whisstock, J. C., Pike, R. N., Jin, L., Skinner, R., Pei, X. Y., Carrell, R. W., and Lesk, A. M. (2000) Conformational changes in serpins: II. The mechanism of activation of antithrombin by heparin, *J. Mol. Biol. 301*, 1287−1305.
9. Kim, J., Lee, K. N., Yi, G. S., and Yu, M. H. (1995) A thermostable mutation located at the hydrophobic core of α_1-antitrypsin suppresses the folding defect of the *Z*-type variant, *J. Biol. Chem. 270*, 8597−8601.
10. Fitton, H. L., Skinner, R., Dafforn, T. R., Jin, L., and Pike, R. N. (1998) The N-terminal segment of antithrombin acts as a steric gate for the binding of heparin, *Protein Sci. 7*, 782−788.
11. Erpel, T., Superti-Furga, G., and Courtneidge, S. A. (1995) Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions, *EMBO J. 14*, 963−975.
12. Ersdal-Badju, E., Lu, A., Peng, X., Picard, V., Zendehrouh, P., Turk, B., Bjork, I., Olson, S. T., and Bock, S. C. (1995) Elimination of glycosylation heterogeneity affecting heparin affinity of recombinant human antithrombin III by expression of a β-like variant in baculovirus-infected insect cells, *Biochem. J. 310*, 323−330.
13. McKay, E. J. (1981) A simple two-step procedure for the isolation of antithrombin III from biological fluids, *Thromb. Res. 21*, 375−382.
14. Olson, S. T., Bjork, I., and Shore, J. D. (1993) Kinetic characterization of heparin-catalyzed and -uncatalyzed inhibition of blood coagulation proteinases by antithrombin, *Methods Enzymol. 222*, 525−559.
15. Huntington, J. A., Olson, S. T., Fan, B., and Gettins, P. G. W. (1996) Mechanism of heparin activation of antithrombin. Evidence for reactive center loop preinsertion with expulsion upon heparin binding, *Biochemistry 35*, 8495−8503.
16. Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D., and Goldsmith, E. J. (1992) Structural basis of latency in plasminogen activator inhibitor-1, *Nature 355*, 270−273.
17. Lomas, D. A., Evans, D. L., Finch, J. T., and Carrell, R. W. (1992) The mechanism of *Z* α_1-antitrypsin accumulation in the liver, *Nature 357*, 605−607.
18. Huntington, J. A., Pannu, N. S., Hazes, B., Read, R. J., Lomas, D. A., and Carrell, R. W. (1999) A 2.6 Å structure of a serpin polymer and implications for conformational disease, *J. Mol. Biol. 293*, 449−455.
19. Dunstone, M. A., Dai, W., Whisstock, J. C., Rossjohn, J., Pike, R. N., Feil, S. C., Le Bonniec, B. F., Parker, M. W., and Bottomley, S. P. (2000) Cleaved antitrypsin polymers at atomic resolution, *Protein Sci. 9*, 417−420.
20. Kwon, K. S., Kim, J., Shin, H. S., and Yu, M. H. (1994) Single amino acid substitutions of α_1-antitrypsin that confer enhancement in thermal stability, *J. Biol. Chem. 269*, 9627−9631.
21. Bruce, D., Perry, D. J., Borg, J. Y., Carrell, R. W., and Wardell, M. R. (1994) Thromboembolic disease due to thermolabile conformational changes of antithrombin Rouen-VI (187 Asn → Asp), *J. Clin. Invest. 94*, 2265−2274.
22. Elliott, P. R., Abrahams, J. P., and Lomas, D. A. (1998) Wild-type α_1-antitrypsin is in the canonical inhibitory conformation, *J. Mol. Biol. 275*, 419−425.
23. Elliott, P. R., Lomas, D. A., Carrell, R. W., and Abrahams, J. P. (1996) Inhibitory conformation of the reactive loop of α_1-antitrypsin, *Nat. Struct. Biol. 3*, 676−681.
24. Skinner, R., Abrahams, J. P., Whisstock, J. C., Lesk, A. M., Carrell, R. W., and Wardell, M. R. (1997) The 2.6 Å structure of antithrombin indicates a conformational change at the heparin binding site, *J. Mol. Biol. 266*, 601−609.
25. Meagher, J. L., Huntington, J. A., Fan, B., Gettins, P. G. (1996) Role of arginine 132 and lysine 133 in heparin binding to and activation of antithrombin, *J. Biol. Chem.* 271, 29353−29358.

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