Preparative Induction and Characterization of L-Antithrombin: A Structural Homologue of Latent Plasminogen Activator Inhibitor-1[†]

Mark R. Wardell,*[,‡] Wun-Shaing W. Chang, David Bruce,[§] Richard Skinner, Arthur M. Lesk, and Robin W. Carrell

Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, United Kingdom

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ABSTRACT: The inhibitory mechanism of the serpin family of serine protease inhibitors is characterized by a remarkable degree of conformational flexibility. Various conformational states have been elucidated by X-ray crystallography and indicate that the inhibitory loop, the central A-β-sheet, and the outside edge of the C-β-sheet are particularly mobile. However, no crystal structure of a serpin−enzyme complex is yet available, and the likely nature of the protease-complexed serpin remains for biochemical and biophysical researchers to examine. Here, we show that the biochemical induction of the latent state of antithrombin is slow relative to polymer formation, and infer that this may reflect structural features that are important for the regulation of the initial docking and subsequent locking of serpins with cognate proteases. L-Antithrombin was induced by incubation of native antithrombin at 60 °C for 10 h in the presence of citrate to prevent polymerization. L-Antithrombin was more stable to denaturation by both heat and urea than native antithrombin. Whereas native antithrombin formed binary complexes with synthetic peptide homologues of the inhibitory loop, biochemically induced L-antithrombin did not, indicating that the inhibitory loop of L-antithrombin is probably fully inserted into the A-β-sheet as in the crystal structure. This was confirmed by limited proteolysis studies which demonstrated that the inhibitory loop of L-antithrombin could not be cleaved by five proteases which do cleave the loop of native antithrombin. The limited proteolysis studies also indicated that the "gate" region (residues 236−248) of the biochemically induced L-antithrombin was in a conformation substantially different from that of the native antithrombin. This again is similar to L-antithrombin in the crystal structure in which the gate has "opened" away from the body of the molecule by a rotation of 24° to facilitate the relocation of strand 1C from its ordered position in the C-β-sheet to a disordered surface loop. At 60 °C in the absence of citrate, antithrombin (and other serpins) rapidly polymerizes. In the presence of citrate, the formation of L-antithrombin is slow and increases with time, indicating that the inhibition of polymer formation by citrate allows the time necessary for the much slower formation of the L form. We therefore suggest that L-antithrombin formation is a two-step process: an initial rapid conformational change, probably including partial incorporation of the reactive loop into the A-sheet (as in the active molecule in the crystal structure) and displacement of s1C from the C-β-sheet which supports polymer formation, and a much slower transition to complete loop insertion within the A-β-sheet. It is likely that both the first rapid transitional step and the structural features that impose resistance to the second more extensive conformational change reflect the optimization of the unique inhibitory function in the serpins.

The hemostatic systems of coagulation, fibrinolysis, complement activation, and inflammation are regulated by the relative activities of serine proteases and their serpin inhibitors (Travis & Salvesen, 1983). The inhibitory reaction of the serpins exploits a remarkable degree of conformational flexibility, which results in the trapping of cognate proteases in very tight, almost irreversible complexes. There is much interest in the nature of their conformational states as serpins initially dock with target proteases and then subsequently form locked complexes with them in a 1:1 stoichiometric ratio.

Crystal structures of several different serpins have given static representations of various conformational states, but no structure of a serpin complexed with its cognate protease is yet available to indicate the inhibitory form. From the structures available, it is apparent that certain features of the serpin molecule are particularly mobile, including the reactive loop, the central A-β-sheet, and the C-β-sheet [all secondary structure elements are as defined in Carrell *et al.* (1994)]. This is clearly demonstrated by comparison of the structural extremes of ovalbumin (a noninhibitory serpin; Stein *et al.*, 1990) and latent plasminogen activator inhibitor-1 (PAI-1)[1] (Mottonen *et al.*, 1992). In the former, the reactive loop consists of a three-turn helix held by two peptide stalks some 7−8 Å away from the body of the molecule in a solvent-

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* To whom correspondence should be addressed: Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Campus Box 8231, 660 S. Euclid Ave., St. Louis, MO 63110-1093. Telephone: (314) 747-0725. Fax: (314) 362-7183. E-mail: mrw@patmos.wustl.edu.

[‡]Present address: Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Campus Box 8231, 660 S. Euclid Ave., St. Louis, MO 63110-1093.

[§] Present address: Heart Research Institute, 145−147 Missenden Road, Campertown, Sydney NSW 2042, Australia.

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[1] Abbreviations: PAI-1, plasminogen activator inhibitor-1; L-antithrombin, latent antithrombin; DEAE, diethylaminoethyl; SDS, sodium dodecyl sulfate; TUG, transverse urea gradient; PVDF, poly(vinylidene difluoride); HNE, human neutrophil elastase.

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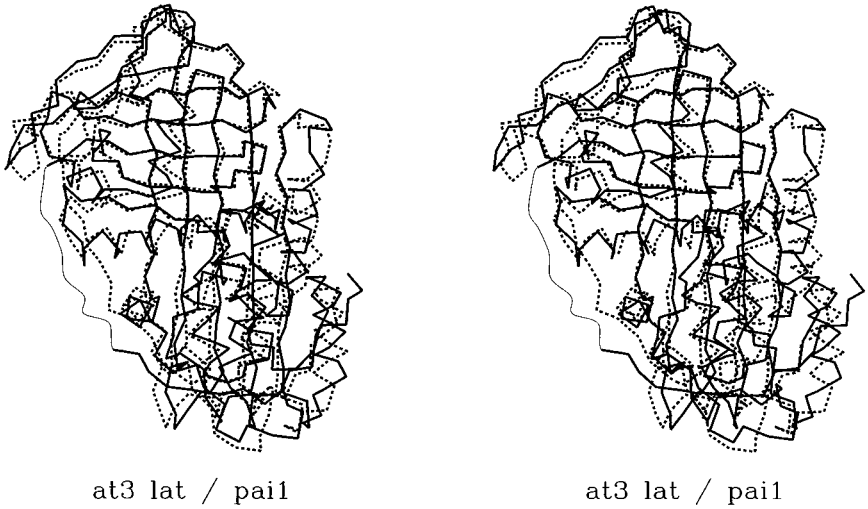

FIGURE 1: Superposition of the structures of L-antithrombin (Carrell *et al*., 1994; solid line) and latent plasminogen activator inhibitor-1 (Mottonen *et al*., 1992; dashed line). Each serpin has undergone an identical conformational rearrangement which includes complete insertion of the reactive loop residues into the A-β-sheet as strand 4A with concomitant release of strand 1C ($P'_8-P'_{11}$; residues 401−404) from the edge of the C-β-sheet. The residues that were in strand 1C, together with $P_3-P'_7$ (residues 391−400) of the reactive loop, now form a large surface loop, the latent loop, here shown on the left side of the structures, which extends from s4B to the bottom of s4A. The latent loop in L-antithrombin is depicted here as a thinner solid line, as no electron density was seen for these residues in the crystal structure.

exposed hydrophilic environment, accessible to proteases. The A-β-sheet in ovalbumin contains five strands, running antiparallel except for central strands 3A and 5A which hydrogen bond in a parallel orientation, and its C-β-sheet contains four strands. In latent PAI-1, a major portion of the reactive loop exists as a β-strand located in the hydrophobic interior of the molecule, inserted as a sixth strand (s4A) of the A-β-sheet, which is now all antiparallel. To facilitate this movement of the reactive loop, the A-β-sheet must open and the first (edge) strand of the C-β-sheet is totally displaced, resulting in only a three-stranded C-sheet.

Antithrombin can adopt a state identical in topology to that of latent PAI-1 (Figure 1) as demonstrated in our recent crystal structure (Wardell *et al*., 1993; Carrell *et al*., 1994; Skinner *et al*., 1997), and so we termed it L-antithrombin (L for latent). This conformation is therefore not exclusive to PAI-1, and is likely to be observed in other serpins as well. However, whereas PAI-1 spontaneously adopts the inactive latent conformation once it is removed from vitronectin, which serves to hold it in a functionally active form (Wiman *et al*., 1988; Andreasen *et al*., 1990), the conversion of antithrombin to the latent state is not normally spontaneous (being seen only in a thermolabile natural antithrombin variant associated with thromboembolic disease; Bruce *et al*., 1994). Another difference between the latent states of PAI-1 and antithrombin is the fact that inhibitory activity can be regained from the former after denaturation and refolding (Hekman & Loskutoff, 1985), whereas it cannot be from the latter (results presented here) or indeed from native antithrombin (Fish *et al*., 1985). In both these serpins, the latent state (and that in which the reactive loop has been proteolytically cleaved) is more stable than the active metastable conformation secreted into plasma. The secreted forms of these and other serpins retain some of the energy of folding to drive the inhibitory reaction (Creighton, 1992; Goldsmith & Mottonen, 1994).

There is likely a conformational continuum between the two extremes exhibited by ovalbumin and latent PAI-1 with the inhibitory state(s) existing somewhere between them. An intermediate form is seen in the "partially activated" antithrombin structure (Carrell *et al*., 1994; Schreuder *et al*., 1994; Skinner *et al*., 1997) where two residues of the reactive loop have been inserted into the A-β-sheet which is seen to be halfway open. It is likely, however, that the reactive loops of serpins locked stably with proteases are inserted into the A-β-sheet to a greater extent than that in the active antithrombin structure (Björk *et al*., 1993; Shore *et al*., 1995). To learn more about the properties of different states within the serpin conformational continuum, we have developed a biochemical method, based on moderate heating at 60 °C, which causes the induction of antithrombin to the latent form. The change to the latent structure proceeds through an initial conformational transition which promotes the rapid formation of polymers. The inclusion of citrate during heating prevents polymerization and allows the much slower conversion to the latent state. We also show here that a previously reported method using partial denaturation by dilute guanidine hydrochloride to induce latent antithrombin (Carrell *et al*., 1991) in fact results in the predominant formation of polymers, and no L-antithrombin.

MATERIALS AND METHODS

Purification of Antithrombin. Antithrombin was isolated from 2 L of fresh frozen plasma using heparin−Sepharose as previously described (McKay, 1981; Bruce *et al*., 1994) with residual heparin being removed by DEAE-Sephadex A-50 or Poros-Q (Perseptive Biosystems, Cambridge, MA) anion exchange chromatography. Most samples for this study were prepared fresh for use, but if antithrombin was to be stored, it was snap-frozen using liquid nitrogen and stored at −80 °C and then re-passaged over heparin−Sepharose and anion exchange just before use. The concentration of antithrombin was determined with an extinction coefficient $E^{1\%}_{280}$ of 6.5 (Nordenman *et al*., 1977) and its specific activity determined using α-thrombin (a gift of J.-M. Freyssinet) as previously described (Bruce *et al*., 1994). The activities of various antithrombin preparations were also evaluated on the basis of whether they could form SDS-stable complexes with thrombin. For this, antithrombin (final

concentration of 1 mg/mL) was incubated with human α-thrombin at a 2:1 molar ratio in 20 mM Tris-HCl at pH 8.0 for 10−20 s at 23 °C. All reactions were terminated by the addition of SDS sample incubation buffer (62.5 mM Tris-HCl, 3% SDS, 10% glycerol, and 0.1% bromophenol blue at pH 6.8) and then immediately snap-frozen in liquid nitrogen and stored at −80 °C until being heated to 95 °C for 2 min just prior to separation by SDS−polyacrylamide gel electrophoresis.

Guanidine Hydrochloride Treatment of Antithrombin. Attempts to produce L-antithrombin by partial guanidine hydrochloride denaturation according to the previously reported method (Carrell *et al*., 1991) utilized freshly prepared native antithrombin at final concentrations ranging between 0.4 and 0.8 mg/mL, incubated with final concentrations of guanidine hydrochloride varying between 0.7 and 1.1 M, in 50 mM Tris-HCl and 50 mM NaCl at pH 7.6 and 4 °C for times varying between 12 and 24 h. Heparin (Grampian Enzymes, Arthrath, U.K.) at concentrations equimolar with respect to that of antithrombin was included in some experiments. At the end of the incubation period, the antithrombin sample was diluted from 0- to 4-fold with dialysis buffer and then dialyzed into either 50 mM Na_2HPO_4 and 10 mM NaCl at pH 7.2 or 50 mM Tris-HCl and 10 mM NaCl at pH 7.2, and then finally into 20 mM Tris-HCl at pH 8.0. It was then applied to heparin−Sepharose where the L-antithrombin was expected to have a reduced heparin affinity; however, only polymeric and no L-antithrombin was obtained from the material eluting under the peak with a reduced heparin affinity.

Preparation of Latent Antithrombin. Latent antithrombin was initially prepared by incubating freshly prepared native antithrombin (final concentration of 5.4 mg/mL) with 0.55 M trisodium citrate and 10 mM Tris-HCl at pH 7.4 and 60 °C for 10 h. We subsequently found that the L-antithrombin yield could be increased from ∼15 to 50−60% by decreasing the concentration of citrate to 0.25 M and increasing the incubation time at 60 °C to 15 h. Final antithrombin concentrations between 1 and 5 mg/mL appear to make no difference in the final yield of L-antithrombin. The L-antithrombin can be obtained in pure form as an early-eluting, low-affinity fraction on heparin−Sepharose chromatography. The time of incubation at 60 °C may also need to be optimized between 5 and 15 h for different batches of antithrombin.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis was performed using 10 to 20% (w/v) linear polyacrylamide gradient gels according to the method of Laemmli (1970) but using 2-amino-2-methyl-1,3-propanediol instead of Tris base. Nondenaturing polyacrylamide gel electrophoresis was a modification of the method of Goldenberg (1989) and was performed at pH 8.9 in 7.5 to 15% gradient gels as previously described (Bruce *et al*., 1994). Transverse urea gradient (TUG) polyacrylamide minigels were prepared with the same buffer system as that used for nondenaturing polyacrylamide gel electrophoresis using a linear gradient from 0 to 8 M urea in 7.5% (w/v) acrylamide (Goldenberg, 1989; Mast *et al*., 1991, 1992; Bruce *et al*., 1994).

Synthetic Peptide Annealing Studies. Antithrombin at a final concentration of 1 mg/mL in 50 mM Tris-HCl and 50 mM NaCl at pH 8.0 was incubated at 37 °C for 24 h with 100-fold molar excess of reactive loop homologue peptides either 6 or 12 amino acids in length. The 6mer peptide represented the $P_{14}-P_9$ [nomenclature of Schechter and Berger (1967); Ac-SEAAAS-OH] and the 12mer peptide the $P_{14}-P_3$ (Ac-SEAAASTAVVIA-OH) residues of the inhibitory loop of antithrombin. After incubation, excess peptides were removed by concentration/dialysis against 20 mM Tris-HCl at pH 7.4 in a Centricon-30 cell (Amicon Inc., Beverly, MA), and the presence of the binary complex was assessed by nondenaturing polyacrylamide gel electrophoresis.

Limited Proteolysis of Antithrombin. Native and L-antithrombin were used at a concentration of 1 mg/mL unless otherwise stated. Human neutrophil elastase was isolated from human empyemic exudate using a modification of the methods of Baugh and Travis (1976) and Martodam *et al*. (1979) and was incubated with native and L-antithrombin in 20 mM Tris-HCl at pH 8.0 at a ratio of 1:250 (w/w) for 2 h at 37 °C. Trypsin and chymotrypsin were incubated with native antithrombin at a ratio of 1:5 (w/w) and with L-antithrombin at a ratio of 1:50 (w/w) in 20 mM Tris-HCl and 20 mM $CaCl_2$ at pH 8.5 for 90 min at 37 °C. Subtilisin was incubated with native and L-antithrombin at a ratio of 1:20000 (w/w) in 50 mM Tris-HCl and 50 mM $CaCl_2$ at pH 7.4 for 2 h at 37 °C. Thermolysin was incubated with native and L-antithrombin (each at a concentration of 0.2 mg/mL) at a ratio of 1:100 (w/w) in 20 mM Tris-HCl and 2 mM $CaCl_2$ at pH 7.2 for 2 h at 37 °C. All the reactions were terminated by the addition of SDS sample incubation buffer (see above) and then the mixtures immediately snap-frozen in liquid nitrogen and stored at −80 °C until being heated to 95 °C for 2 min and separated by SDS−polyacrylamide gel electrophoresis. Results were identical when digestion was first stopped by the addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM. The gels were then electrotransferred onto a PVDF membrane (ProBlot, Applied Biosystems, Cheshire, U.K.), and the amino terminus of each proteolytic fragment of antithrombin was determined by Edman degradation.

Denaturation and Refolding of L-Antithrombin. Native and L-antithrombin (each at a final concentration of 1 mg/mL) were denatured by incubation at 4 °C for 8 h in 6 M GdHCl and 50 mM Tris-HCl at pH 7.6 both with and without 100 mM dithiothreitol. Subsequently, it was diluted to 0.15 mg/mL in 4 M GdHCl and 50 mM Tris-HCl at pH 7.6 and dialyzed at 4 °C sequentially against the same buffer containing lower GdHCl concentrations on the order of 3, 2, 1, and finally 0 M. When denaturation included reduction, the redox potential of the sample solution was controlled to allow disulfide exchange by including 10 mM reduced and 1 mM oxidized glutathione in the dialysis buffers. Oxygen was removed from some of the dialysis buffers by degassing and purging with nitrogen for 2 h prior to beginning the dialysis of the sample and then continued purging with nitrogen gas during dialysis.

Coordinates and Calculations. Coordinates of antithrombin were produced in this laboratory (Carrell *et al*., 1994; Skinner *et al*., 1997) and have been deposited in the Brookhaven Protein Data Bank (1ANT and 2ANT). Coordinates of PAI-1 were given to us by E. J. Goldsmith (University of Texas Southwestern Medical Center, Dallas, TX) and are available from the Brookhaven Protein Data Bank (1AEA). Superimpositions and comparisons of the structures of latent antithrombin and latent PAI-1 and of

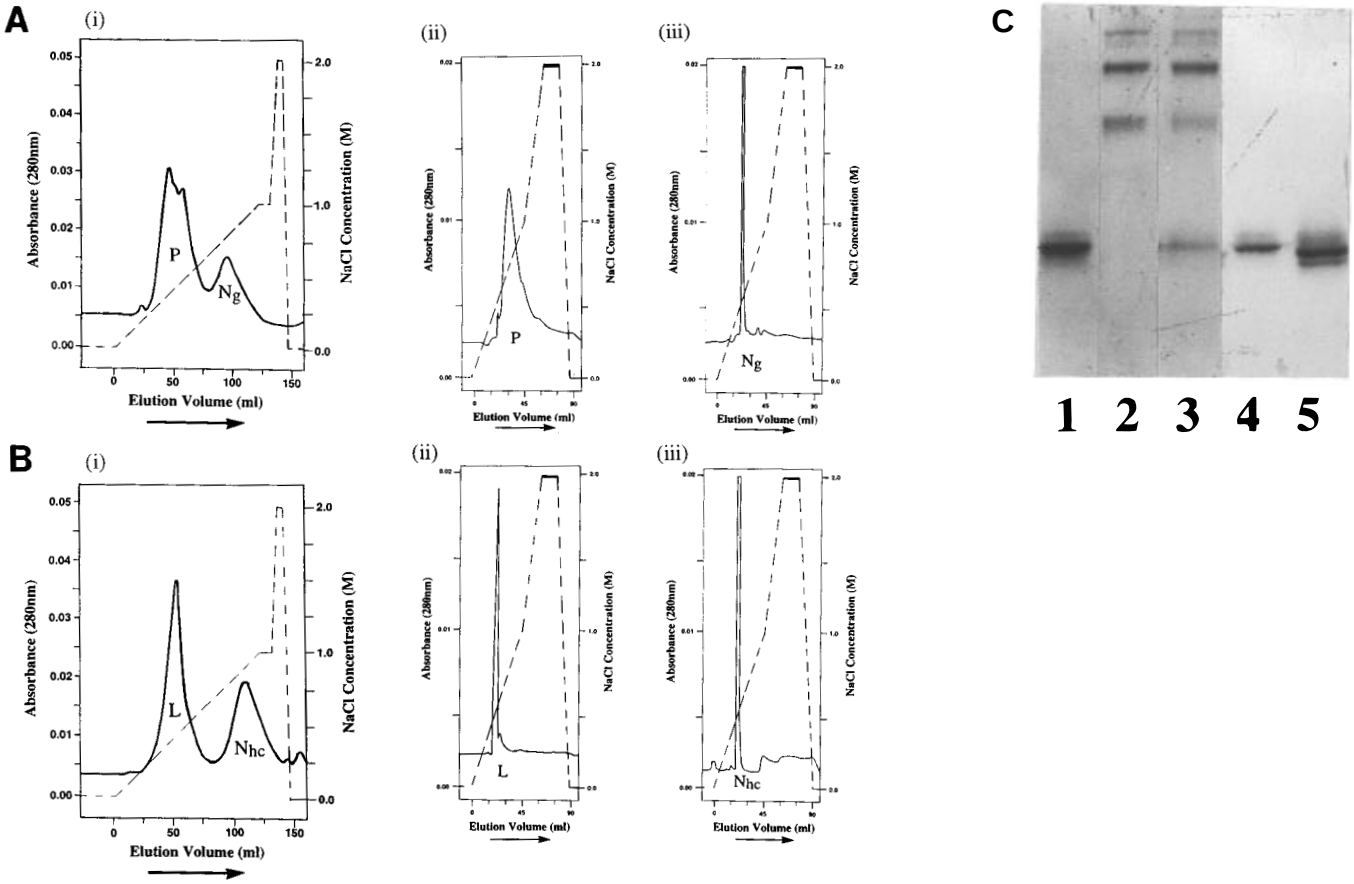

FIGURE 2: Preparation and isolation of L-antithrombin. (A) Fractionation of human plasma antithrombin treated with guanidine hydrochloride. The early-eluting material, with a lower heparin affinity (labeled P in panel i of part A), was shown by both anion exchange chromatography (panel ii of part A) and nondenaturing polyacrylamide gel electrophoresis (lane 3 of part C) to contain predominantly polymers, and no L-antithrombin could be obtained. Peak N_g in panels i and ii of part A represents native antithrombin remaining after guanidine hydrochloride treatment. (B) Fractionation of human plasma antithrombin heated for 10 h at 60 °C in the presence of 0.25 M sodium citrate. Peak L, with a lower heparin affinity in panel i of part B, was shown by both anion exchange chromatography (panel ii of part B) and nondenaturing polyacrylamide gel electrophoresis (lane 5 of part C) to be exclusively monomeric. This material also has a more anodal electrophoretic mobility (lane 5 of part C) than native antithrombin (lanes 1 and 4 of part C), a characteristic feature of L-antithrombin. Peak N_{hc} in panels i and iii of part B represents native antithrombin remaining after heat treatment in the presence of citrate. All dashed lines represent NaCl gradients with the NaCl concentrations indicated on the right of each panel. (C) Nondenaturing gradient polyacrylamide gel electrophoresis (7.5 to 15%): lane 1, freshly isolated plasma antithrombin; lane 2, antithrombin heated at 60 °C for 60 min in the absence of citrate; lane 3, antithrombin treated with 0.9 M guanidine hydrochloride at 4 °C for 16 h; lane 4, native antithrombin obtained from peak N_{hc} from panels i and iii of part B after heat treatment at 60 °C for 10 h in the presence of 0.25 M sodium citrate; and lane 5, L-antithrombin obtained from peak L from panels i and ii of part B after heat treatment at 60 °C for 10 h in the presence of 0.25 M sodium citrate. The cathode (−) is at the top, and the anode (+) is at the bottom.

native and latent antithrombin were carried out using the 1ANT coordinates with programs written by one of us (Lesk, 1994).

RESULTS

Preparative Induction of Latent Antithrombin. The previously reported method for inducing latent antithrombin with partial denaturation with guanidine hydrochloride (Carrell *et al*., 1991) produces predominantly polymers (panels i and ii of part A and lane 3 of part C of Figure 2) and no L-antithrombin. An alternative method for the induction of L-antithrombin was deduced from the findings of earlier work (Holleman *et al*., 1977; Hoffman, 1989) associated with the stabilization of antithrombin being prepared for supplementation therapy. The inclusion of 0.5 M sodium citrate was shown to reduce substantially the loss of anticoagulant activity in antithrombin during pasteurization treatment (60 °C for 10 h) to inactivate viruses (Hoffman, 1989). However, it was noted that these conditions did not prevent the loss of all inhibitory activity and often altered the electrophoretic properties of the antithrombin preparation (Wickerhauser *et al*., 1979). The major effect of citrate and other stabilizers was shown by Busby *et al*. (1981) to be the prevention of antithrombin aggregation, and we wondered whether this treatment could be adapted to the selective induction of L-antithrombin.

Freshly isolated human plasma antithrombin subjected to pasteurization at 60 °C for 10 h in the presence of 0.55 M sodium citrate produced a significant fraction of the antithrombin (∼15−20%) converting from a high- to a low-affinity heparin state (panel i of part B of Figure 2). Whereas antithrombin incubated under the same conditions, but in the absence of citrate, resulted in only polymers (Figure 2C, lane 2), the presence of citrate completely inhibited polymer formation (panels ii and iii of part B and lanes 4 and 5 of part C of Figure 2).

The early-eluting fraction with reduced heparin affinity was shown to be monomeric (panel ii of part B and lane 5 of part C of Figure 2), and it migrated more anodally on both nondenaturing polyacrylamide gel electrophoresis (lane

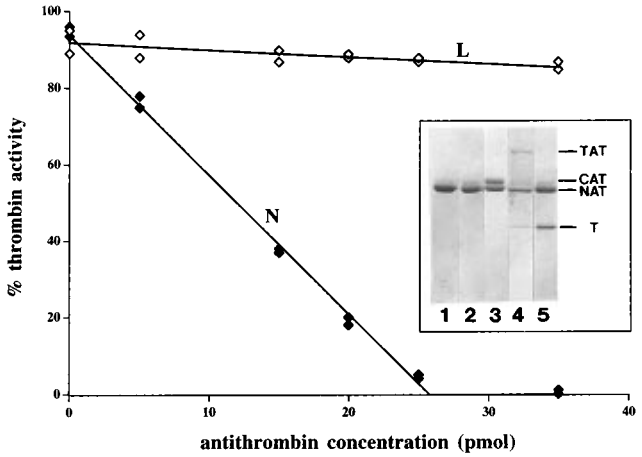

FIGURE 3: Specific inhibitory activity of L-antithrombin toward thrombin. L-Antithrombin (◇) and native antithrombin (◆) were incubated at the indicated concentrations with 20 pmol of active α-thrombin for 2 h at 23 °C. Residual thrombin activity was then determined from the release of *p*-nitroaniline from the substrate S-2288. Each measurement was performed in triplicate, and two inhibitory titrations are shown for each antithrombin conformation: inset, thrombin−antithrombin complex (TAT) formation determined by SDS−polyacrylamide gel electrophoresis; lane 1, L-antithrombin; lane 2, native antithrombin (NAT); lane 3, mixture of native antithrombin and reactive loop-cleaved antithrombin (CAT); lane 4, 1 μg of thrombin (T) and 4.3 μg of native antithrombin (note the TAT complex); and lane 5, 1 μg of thrombin (T) and 4.3 μg of L-antithrombin (note both the absence of the TAT complex and the fact that L-antithrombin does not act as a substrate for α-thrombin).

5 of part C of Figure 2) and isoelectric focusing (data not shown). Whereas the antithrombin with normal heparin affinity (peak N_{hc} in panels i and iii of part B of Figure) had normal thrombin inhibitory activity (Figure 3) and could form SDS-stable complexes with thrombin (lane 4 of the Figure 3 inset), that with reduced heparin affinity (peak L in panels i and ii of part B of Figure 2) was totally noninhibitory (Figure 3) and did not form SDS-stable complexes with thrombin (lane 5 of the Figure 3 inset). In addition to being noninhibitory, the peak L antithrombin was not a substrate for thrombin (lane 5 of the Figure 3 inset). This lack of cleavage by thrombin was confirmed by sequence analysis, which gave as a single sequence the amino terminus of antithrombin (His-Gly-Ser-Pro-Val-Asp-Ile-X-Thr-Ala-Lys). We therefore refer to this protein as the L form for latent antithrombin because, as we now show, its properties suggest it is structurally analogous to the crystallographically determined L-antithrombin and latent PAI-1.

At the minimum citrate concentration of 0.2 M required to inhibit polymerization, we found that the yield of L-antithrombin was increased. The time of pasteurization also influences the L-antithrombin yield as shown in Figure 4. However, at times longer than 15 h in the presence of 0.25 M citrate, or if the antithrombin used to prepare the L state had been previously stored inappropriately, we observed the appearance of additional, even more anodal electrophoretic species (data not shown). The rest of this study was performed with L-antithrombin prepared from freshly isolated plasma antithrombin that had not been frozen, by incubation at 60 °C for 15 h in the presence of 0.25 M sodium citrate. If older antithrombin that has been frozen is used as the starting material, then the citrate concentration should be increased to 0.3−0.35 M with incubation at 60 °C for 10−12 h, but this will decrease the yields of L-antithrombin (data not shown).

Stability of Latent Antithrombin. Latent antithrombin was found to be almost as thermostable as cleaved antithrombin (data not shown) with a tendency to polymerize only at 100 °C, when subjected to classical heat stability analysis (Carrell & Owen, 1985; Pemberton *et al*., 1988; Stein *et al*., 1989; Hopkins *et al*., 1993). L-Antithrombin also exhibited a stability similar to that of cleaved antithrombin on transverse urea gradient (TUG) gel electrophoresis with neither of them demonstrating the characteristic unfolding transition of native antithrombin (Mast *et al*., 1991; Figure 5). This dramatic difference in stability between native and L-antithrombin when they are exposed to urea is seen clearly in Figure 5F, which represents an equal mixture of the two conformations.

Peptide Annealing. When the 12mer synthetic homologue loop peptide (P_{14}−P_3) is incubated with antithrombin at 37 °C, it is presumed to anneal within the central A-β-sheet in the position of strand 4A in the cleaved crystal structure (Schultze *et al*., 1990, 1992; Björk *et al*., 1992a,b). The resulting binary complex can be distinguished from native antithrombin on nondenaturing polyacrylamide gel electrophoresis as it migrates more anodally due to the acquisition of two additional negative charges from the synthetic peptide (Bruce *et al*., 1994; Chang *et al*., 1997). The 12mer peptide apparently was unable to anneal to L-antithrombin, as no electrophoretic shift was observed even after times of incubation with the peptide that resulted in all of the native antithrombin becoming a binary complex (lanes 1−4 of Figure 6). Furthermore, L-antithrombin does not react with the smaller 6mer homologue loop peptide (P_{14}−P_9, lane 6 of Figure 6), whereas the 6mer peptide induces native antithrombin to polymerize (Chang *et al*., 1997; lane 5 of Figure 6). Together, these results indicate that under the experimental conditions employed here, using a 100-fold molar excess of peptide over antithrombin, homologue loop peptides do not appear to react with L-antithrombin, consistent with the hypothesis that their most likely point of interaction, the A-β-sheet, is already occupied by the reactive loop.

Limited Proteolysis. If the reactive loop is inserted in the A-sheet to a large degree in L-antithrombin, then it should not be susceptible to proteolytic attack. Cleavage within the reactive loop of native antithrombin causes a characteristic shift in its electrophoretic mobility on nonreducing SDS−polyacrylamide gels to a position with a larger apparent size. This can be seen in Figure 7 using the enzymes human neutrophil elastase (lane 2), trypsin (lane 5), chymotrypsin (lane 7), subtilisin (lane 9), and thermolysin (lane 11). The positions of cleavage within the loop were determined by sequence analysis and are given in Table 1. The proteolytic cleavage patterns of L-antithrombin were found to be markedly different as shown in Figure 7 for human neutrophil elastase (lane 4), trypsin (lane 6), chymotrypsin (lane 8), subtilisin (lane 10), and thermolysin (lane 12). Amino-terminal sequencing of the fragments released indicated that three regions, different from the reactive loop, had become proteolytically susceptible in L-antithrombin; (1) the amino terminus, (2) the loop between the third and fourth strands of the C-β-sheet described by Tucker *et al*. (1995) as the "gate" region of serpins (residues 236−248 and shown in Figure 8A), and (3) the loop between s4B and helix G. The

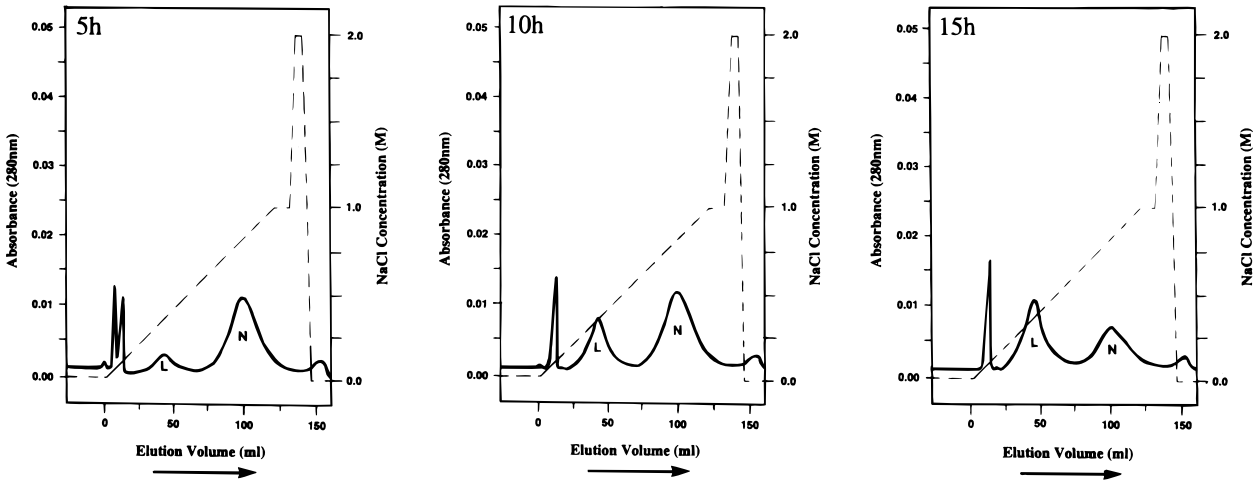

FIGURE 4: Effect of incubation time on the yield of L-antithrombin. Antithrombin (2 mg/mL) was incubated at 60 °C in the presence of 0.25 M sodium citrate for the indicated times. After incubation, the yield of L-antithrombin was monitored by heparin−Sepharose chromatography.

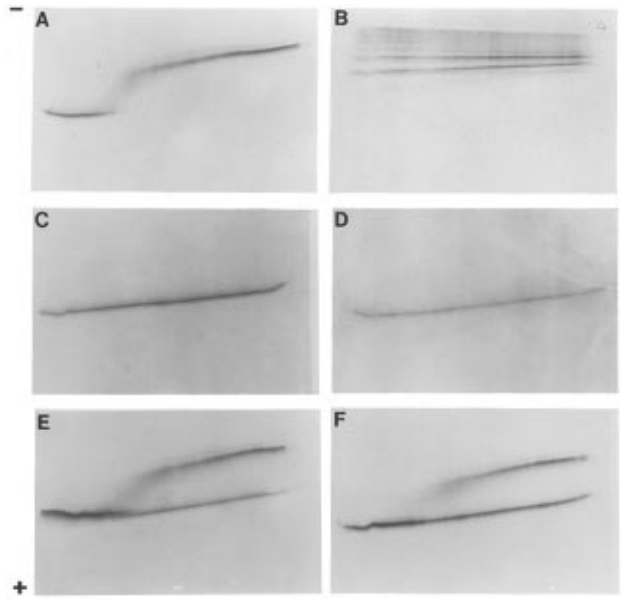

FIGURE 5: Transverse urea gradient (TUG) polyacrylamide gel electrophoresis of different antithrombin conformations: (A) 50 μg of native antithrombin, (B) 50 μg of antithrombin polymers, (C) 50 μg of reactive loop-cleaved antithrombin, (D) 50 μg of L-antithrombin, (E) a mixture of 50 μg each of native and reactive loop-cleaved antithrombin, and (F) a mixture of 50 μg each of native and L-antithrombin. A linear urea gradient from 0 to 8 M runs from left to right in each gel.

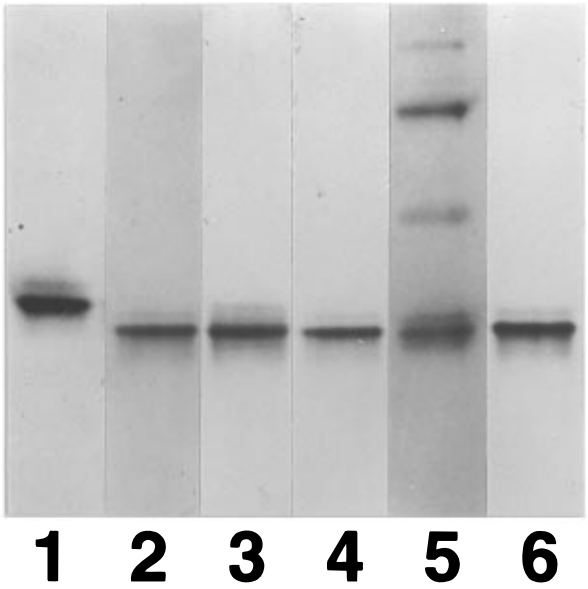

FIGURE 6: Nondenaturing polyacrylamide gel electrophoresis of native and L-antithrombin with and without reactive loop homologue peptides annealed in the A-β-sheet: lane 1, 5 μg of native antithrombin; lane 2, 5 μg of native antithrombin with the synthetic 12mer peptide representing residues $P_{14}-P_3$ of the reactive loop annealed in the A-β-sheet (the more anodal migration of the binary complex occurs because the homologue loop peptide carries a net charge of −2); lane 3, 5 μg of L-antithrombin (note that the electrophoretic mobility of L-antithrombin is the same as that of the native binary complex); lane 4, 5 μg of L-antithrombin incubated for 24 h at 37 °C with the synthetic homologue loop 12mer peptide; lane 5, 5 μg of native antithrombin incubated for 24 h at 37 °C with the homologue loop 6mer peptide representing residues $P_{14}-P_9$ of the reactive loop; and lane 6, 5 μg of L-antithrombin incubated as above with the homologue loop 6mer peptide.

cleavage locations created in L-antithrombin by these proteases are given in Table 1.

The gate region within L-antithrombin appeared to be particularly susceptible and was attacked by all the proteases that were used except human neutrophil elastase. A superimposition of this region (Figure 8) of the active and L state conformations from the crystal structure indicates that residues 236−248 of L-antithrombin are significantly displaced relative to those of native antithrombin. The rms deviation of the C_α atoms for these 13 residues is 4.58 Å compared to 1.0 Å for all residues between 45 and 432. Eleven of these residues (238−248) deviate by more than 2.8 Å with a minimum deviation of 2.82 Å (residue 248) to a maximum of 8.68 Å (residue 245). Geometrically, these residues in the L form have undergone a rotation of 24° and a small translation of 0.17 Å with respect to the corresponding residues in the native structure (Figure 8B). The axis of this rotation is roughly perpendicular to both the plane of the A-sheet and the mean strand direction and makes an angle of about 45° with the plane of the A-sheet. The limited proteolysis results clearly indicate that the gate region in the biochemically produced L-antithrombin is also significantly different from its counterpart in native antithrombin.

Denaturation and Refolding of L-Antithrombin. Native and L-antithrombin were denatured with 6 M GdHCl, both with and without a reducing agent (100 mM dithiothreitol).

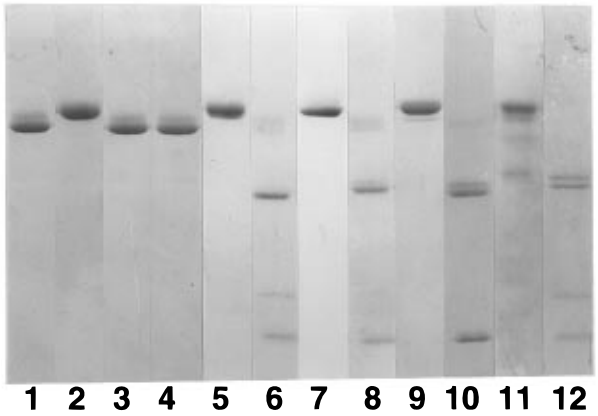

FIGURE 7: Limited proteolysis of native and L-antithrombin assessed by nonreducing SDS−polyacrylamide gel electrophoresis: lane 1, 5 μg of native antithrombin; lane 2, 5 μg of antithrombin cleaved in the reactive loop (P_4-P_3) by human neutrophil elastase; lane 3, 5 μg of L-antithrombin; lane 4, 5 μg of L-antithrombin incubated with human neutrophil elastase; lane 5, 5 μg of antithrombin cleaved in the reactive loop ($P_1-P'_1$) by trypsin; lane 6, 5 μg of L-antithrombin cleaved by trypsin; lane 7, 5 μg of antithrombin cleaved in the reactive loop ($P'_2-P'_3$) by chymotrypsin; lane 8, 5 μg of L-antithrombin cleaved by chymotrypsin; lane 9, 5 μg of antithrombin cleaved in the reactive loop (P_7-P_6) by subtilisin; lane 10, 5 μg of L-antithrombin cleaved by subtilisin; lane 11, 5 μg of antithrombin cleaved in the reactive loop ($P'_1-P'_2$) by thermolysin; and lane 12, 5 μg of L-antithrombin cleaved by thermolysin.

Table 1: Proteolytic Cleavage Positions in Native and L-Antithrombin

protease	native antithrombin	L-antithrombin
HNE[a]	I^{390}-A^{391} (P_4-P_3)[b]	none
trypsin	R^{393}-S^{394} ($P_1-P'_1$)	K^{39}-I^{40}, K^{236}-E^{237}, K^{290}-S^{291}
chymotrypsin	L^{395}-N^{396} ($P'_2-P'_3$)	Q^{38}-K^{39}, L^{238}-F^{239}, Y^{240}-K^{241}
subtilisin	A^{387}-V^{388} (P_7-P_6)	K^{29}-A^{30}, Q^{38}-K^{39}, A^{242}-D^{243}
thermolysin	S^{394}-L^{395} ($P'_1-P'_2$)	E^{237}-L^{238}, (L^{238}-F^{239}, F^{239}-Y^{240}),[c] S^{291}-L^{292}

[a] HNE = human neutrophil elastase. [b] Nomenclature of Schechter and Berger (1967). [c] Minor cleavages.

Each protein (1 mg/mL) was incubated at 4 °C under these conditions for 8 h, after which time it was diluted to a final protein concentration of 0.15 mg/mL and 4 M GdHCl and then subjected to sequential dialysis in progressively reduced concentrations of GdHCl as detailed in Materials and Methods. When denaturation included reduction, the redox potential of the buffer was controlled by the addition of both reduced and oxidized glutathione to allow disulfide exchange during refolding. No inhibitory activity could be regenerated from either initial protein conformation, irrespective of whether it was reduced during denaturation. Therefore, the loss of inhibitory activity in antithrombin that has been converted to the L state is irreversible.

DISCUSSION

In this study, we have defined a biochemical method for the induction of antithrombin to a latent state and provided evidence that its conformation resembles that previously seen in the crystal structure. L-Antithrombin was induced by limited thermal denaturation in the presence of sodium citrate to prevent polymerization. The effect of citrate on stabilizing antithrombin was previously investigated by Busby *et al.* (1981), who found that the temperature at which antithrombin aggregation occurred was increased nearly 20 °C in the presence of 1 M citrate. These workers demonstrated that stabilization of antithrombin by citrate was not due to direct binding to the protein or to its chelating properties, but rather through its lyotropic effects, which modify the strength of intramolecular hydrophobic interactions by influencing the structure of water (von Hippel & Schleich, 1969; von Hippel & Hamabata, 1973). The relative stabilization of antithrombin by other salts correlated well (Busby *et al.*, 1981) with their position in the lyotropic series (von Hippel & Wong, 1965).

Using industrial pasteurization conditions of incubation for 10 h at 60 °C in the presence of 0.55 M citrate, we show that approximately 15−20% of the antithrombin is converted to the L form, with a complete inhibition of polymer formation. The yield of L-antithrombin can be substantially increased (to 50−60%) by lowering the citrate concentration to 0.25 M and by manipulation of the incubation time (Figure 4). The L-antithrombin has a decreased heparin affinity and can be obtained in pure form with heparin−Sepharose chromatography (Figure 2B). It is neither an inhibitor nor a substrate of thrombin, indicating its reactive loop is unavailable for interaction with thrombin and consistent with the loop being fully incorporated into the A-β-sheet, as in the crystal structure. Additional evidence for this was provided in three different ways: (1) stability measurements, (2) peptide annealing experiments, and (3) limited proteolysis.

L-Antithrombin was both more thermostable than native antithrombin and more resistant to denaturation by urea as shown in Figure 5. Whereas native antithrombin demonstrated an unfolding transition at urea concentrations below 3 M, L-antithrombin was resistant to unfolding by urea at concentrations of up to 8 M. In this respect, it resembled reactive loop-cleaved antithrombin in which the loop is fully incorporated into the A-sheet. Homologue loop peptides were also unable to anneal to L-antithrombin; the longer 12mer peptide formed an expected binary complex with native antithrombin, while the shorter 6mer peptide unexpectedly caused it to polymerize (lane 5 of Figure 6: Chang *et al.*, 1997) by an unknown mechanism. The electrophoretic mobility of L-antithrombin was unaffected by either peptide, suggesting that the degree of loop insertion into the A-sheet in L-antithrombin is extensive, thereby preventing access of the synthetic peptides.

Limited proteolysis results were also consistent with the biochemically produced L-antithrombin resembling that of L-antithrombin in the crystal structure. Five proteases that cleaved native antithrombin within the reactive loop failed to cleave L-antithrombin at the same position; however, three other sites in L-antithrombin became more proteolytically susceptible (Table 1). One of these was the region of the molecule known as the gate (Tucker *et al.*, 1995) which is also seen to have moved significantly relative to the rest of the molecule in the crystal structure of L-antithrombin. As shown in Figure 9, when the crystallographically determined structures of L-antithrombin and native antithrombin are superimposed, the gate (residues 233−252) is one of three regions where the C_α atoms of L-antithrombin deviate by more than 5 Å from the corresponding atom positions in native antithrombin. This region is shown in Figure 8 where residues 236−248 in L-antithrombin appear to have opened away from the body of the molecule through a 24° rotation relative to their position in native antithrombin. This

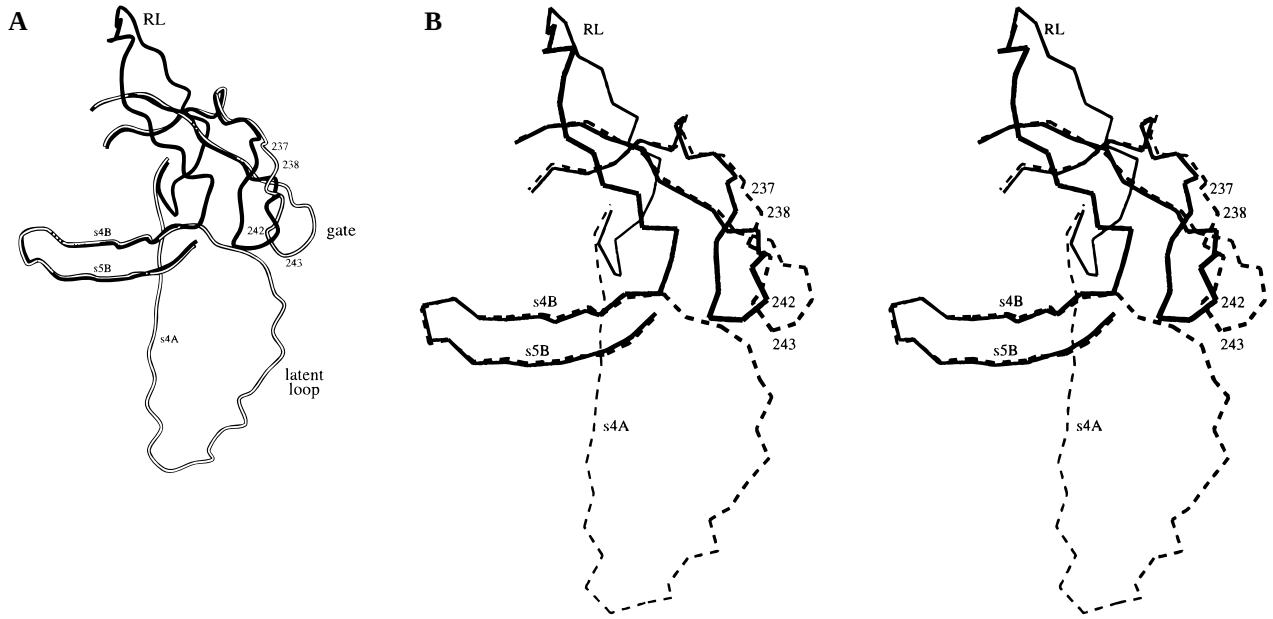

FIGURE 8: Superimposition of the reactive loop (RL) and gate regions of native and L-antithrombin from the crystal structure. (A) Native antithrombin with the exposed reactive loop is shown with the solid line, and the position of the latent loop in L-antithrombin (unfilled line) is indicated. (B) Stereodiagram of the superimposed reactive loop and gate regions of native and L-antithrombin. The gate in L-antithrombin has opened because of a rotation of 24° relative to the equivalent residues (236−248) in native antithrombin.

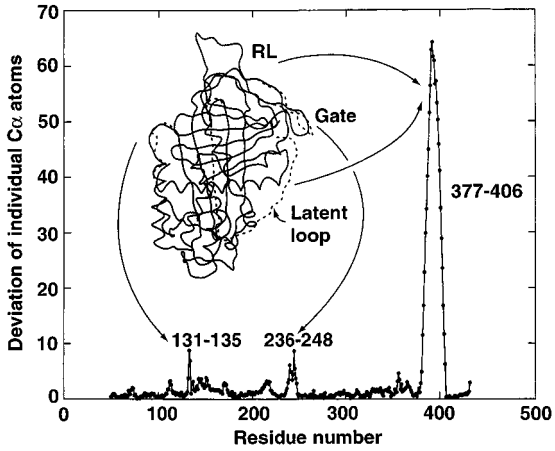

FIGURE 9: Deviation (angstroms) of C_α atoms in L-antithrombin from the corresponding atoms in native antithrombin on which it is superimposed. The three areas of L-antithrombin where the positions of C_α atoms deviate by more than 5 Å from the corresponding atoms in the native structure are indicated by the dashed lines and include the loop between the top of the D-helix and strand 2A of the A-sheet (residues 131−135), the gate region (residues 236−248), and the reactive loop (residues 377−406).

conformational change would be required to allow strand 1C to relocate from its ordered C-sheet position in the native structure to a more disordered position in the latent loop of the L form. The new cleavage positions within this region for four of the five proteases used in this study, trypsin, chymotrypsin, subtilisin, and thermolysin, indicate that the conformation of the gate in the biochemically produced L-antithrombin is also significantly different from that in native antithrombin. This gives a second structural element besides the reactive loop that, in common with L-antithrombin in the crystal structure, is markedly different from its native antithrombin counterpart.

The L state is one of the more stable conformations antithrombin can assume after it has escaped from the local energy minimum in which the metastable active conformation is trapped. Qualitatively, a similar conformational change takes place in serpin structures during their inhibitory reaction with target proteases. In this case, the energy needed to initiate the conformational change is provided by interaction with the protease. The extent of reactive loop incorporation into the A-sheet (Björk *et al*., 1993; Shore *et al*., 1995; Wilczynska *et al*., 1995) and whether strand s1C is displaced (Hopkins *et al*., 1997) in the inhibitory complex have been investigated biochemically, but a more precise picture of these aspects of the serpin inhibitory conformation will ultimately have to await a crystal structure of a serpin−enzyme complex. In the meantime, caution in the interpretation of *in vitro* studies is recommended, as the degree of loop insertion and the mode of intermolecular linkage of polymers, for example, appear to depend upon buffer conditions chosen, as well as other factors (Koloczek *et al*., 1996a,b). However, our present data do suggest some intriguing clues as to the sequence of events that might take place during the inhibitory mechanism. For example, the data are consistent with the formation of L-antithrombin being a two-step process: an initial fast step to a conformation that supports the formation of polymers and a second much slower transition to the full L state if polymerization is prevented. This is demonstrated by the observation that incubation of antithrombin at 60 °C in the absence of citrate results almost completely in the generation of polymers within minutes (Figure 2C), whereas in the presence of citrate (which prevents polymerization), the formation of the L state proceeds over a period of hours, with only about 50% converting to L-antithrombin in 15 h (Figure 4). The reason partial denaturation of antithrombin by guanidine hydrochloride does not yield L-antithrombin, as was previously reported (Carrell *et al*., 1991), but only polymers as shown here, might be the fact that no agent such as citrate was present in the buffer to inhibit polymer formation in the early phases of structural transition and allow the protein the longer time necessary for adoption of the full latent state.

The conformation reached rapidly in the first step of the two-step conformational transition may resemble that of the crystallographically determined active antithrombin structure (Carrell *et al*., 1994; Schreuder *et al*., 1994; Skinner *et al*., 1997), which existed in a dimer which is suggestive of the way polymers might form. This conformation, with partial loop insertion, may closely resemble that adopted rapidly during the docking interaction with protease. Consistent with this is the report by Huntington *et al*. (1995) where the inability of ovalbumin to rapidly insert its reactive loop partially into the A-sheet accounts for the absence of detectable inhibitory activity in that serpin. The subsequent resistance in antithrombin to the more extensive loop insertion of the full L state probably reflects structural features that hold the serpin in the optimal docking conformation long enough for the inhibitory complex between serpin and the cognate enzyme to stabilize. PAI-1 has apparently lost those structural features that provide this resistance against the spontaneous full conversion to the latent state and instead utilizes its association with vitronectin to maintain the active metastable conformation.

The structural elements providing the resistance against the second conformational transition could include the attachment of the amino-terminal portion of the loop to the C-sheet via s1C, but the relative ease with which polymers form suggests that s1C is readily displaced (Chang *et al*., 1997). Two lines of evidence suggest that forces holding the lower half of the A-sheet closed might be more important in this respect. The first comes from the observation that, in the crystal structure of active antithrombin, the lower half of the A-sheet remained closed between strands 3A and 5A while the upper half had apparently opened readily. The second comes from the thermolabile natural variant, antithrombin Rouen-VI, which spontaneously converts to both polymeric and L state conformations at physiological temperature, and significantly faster at fever temperatures (Bruce *et al*., 1994). A single amino acid substitution of aspartate for the conserved asparagine at position 187 destabilizes this variant by breaking a hydrogen bond which is seen in all other serpin crystal structures, thereby indirectly allowing the bottom of the A-sheet to open with the resultant conversion to the inactive forms [see Bruce *et al*. (1994) for a more detailed discussion].

Taken together, the relative ease with which antithrombin and other serpins are induced to polymerize, and their resistance to the formation of the L state, fit a model in which specific structural determinants facilitate the apparent ready ability of the upper portion of the A-sheet to open and allow partial loop insertion while at the same time keeping the lower portion of the A-sheet closed. Antithrombin can now be used as a system complementary to PAI-1 in protein engineering and other experimental approaches to test this hypothesis and locate these key structural elements involved in maintenance of the fragile metastable conformation and which hold the serpins strained like a mousetrap ready to spring shut on target proteases.

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