Identification of an Epitope in Antithrombin Appearing on Insertion of the Reactive-Bond Loop into the A β-Sheet[†]

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ABSTRACT: Previous work has shown that insertion of the reactive-bond loop of antithrombin into the main β-sheet of the inhibitor, the A sheet, leads to exposure of epitopes that are not present in intact antithrombin. Identical epitopes are exposed in antithrombin−proteinase complexes, inferring that the reactive-bond loop is inserted into the A β-sheet also in these complexes. Loop insertion thus presumably is involved in the mechanism of inhibition of target proteinases. In this work, we have identified a linear epitope in bovine antithrombin that reacts with antibodies specific for loop-inserted forms of the inhibitor. This epitope is a hexapeptide sequence comprising residues 342−347, Glu-Asp-Leu-Phe-Ser-Pro, and is located on the surface of the protein just carboxy-terminal of helix I. The Phe residue of this epitope is highly conserved in members of the serpin superfamily and appears to stabilize the region of the epitope in antithrombin and other serpins by interacting with the protein core. The conformational change involving expansion of the A β-sheet following insertion of the reactive-bond loop is presumably transmitted through this Phe residue to the epitope region, thereby rendering this region accessible to antibodies.

Most plasma serine proteinase inhibitors, e.g. α_1-proteinase inhibitor, antithrombin, α_1-antichymotrypsin, α_2-antiplasmin, C1-inhibitor, and plasminogen activator inhibitor-1, belong to the serpin superfamily of proteins. This family also contains several noninhibitory proteins, such as ovalbumin, thyroxine-binding globulin, and angiotensinogen (Carrell & Travis, 1985; Huber & Carrell, 1989). Considerable evidence indicates that the inhibitory serpins share a common mechanism of action appreciably different from that of low-molecular weight protein inhibitors of serine proteinases. Like in the latter type of inhibitors (Laskowski & Kato, 1980; Bode & Huber, 1992), a specific, exposed reactive bond in the serpins is attacked by the target proteinase. However, the serpin inactivation mechanism differs from that of the low-molecular weight inhibitors in involving a substantial conformational change of the inhibitor. This change traps the proteinase in a tight, possibly covalently linked, complex that is only kinetically stable and slowly turns over to inactive, reactive-bond-cleaved inhibitor and free enzyme (Travis & Salvesen, 1983; Carrell & Boswell, 1986; Huber & Carrell, 1989; Bode & Huber, 1992; Olson & Björk, 1994).

The nature of the conformational change involved in proteinase binding by serpins has not yet been elucidated. The X-ray structures of three active serpins, antithrombin, an engineered variant of α_1-antichymotrypsin, and α_1-proteinase inhibitor, have recently been solved (Wei et al., 1994; Schreuder et al., 1994; Carrell et al., 1994; Song et al., 1995). In these structures, the reactive bond is located in a loop on the surface of the protein. This loop is fully exposed in α_1-antichymotrypsin and α_1-proteinase inhibitor, but in the antithrombin structure, two residues at the N-terminal end of the loop are inserted into the major β-sheet of the protein, the A sheet, forming the beginning of a new middle strand of this sheet. Moreover, the polypeptide chain around the reactive bond has a helical conformation in α_1-proteinase inhibitor and α_1-antichymotrypsin but shows no regular secondary structure in antithrombin. It is possible that these deviant features of the antithrombin structure are due to the observed interaction of the reactive-bond loop with a neighboring molecule in the unit cell and that the structures of α_1-proteinase inhibitor and α_1-antichymotrypsin are typical of those of intact serpins. Although the overall three-dimensional structures of the intact serpins are similar to those determined previously for the corresponding inhibitors cleaved at or near the reactive bond (Loebermann et al., 1984; Mourey et al., 1993; Baumann et al., 1991), essential features differ. Notably, in the cleaved inhibitors the N-terminal segment of the reactive-bond loop is no longer on the surface of the protein but is completely inserted as a middle strand of the A β-sheet. The loop is similarly inserted in a latent, inactive form of plasminogen activator inhibitor-1, although the reactive bond is intact (Mottonen et al., 1992). These structures, together with other evidence, have led to proposals that insertion of the reactive-bond loop into the A β-sheet is an essential component of the conformational change involved in trapping of proteinases by serpins (Engh et al., 1990; Schulze et al., 1990; Skriver et al., 1991; Carrell et al., 1991; Björk et al., 1992; Mast et al., 1992; Shore et al., 1995; Wright & Scarsdale, 1995; Lawrence et al., 1995).

We have previously presented experimental evidence that such insertion of the reactive-bond loop into the A β-sheet accompanies formation of complexes between antithrombin and its target proteinases, thrombin and factor Xa (Björk et al., 1993). Antisera specific for reactive-bond-cleaved bovine antithrombin or the bovine antithrombin−thrombin complex

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did not react with intact antithrombin in immunodiffusion analyses but reacted identically with several forms of the inhibitor in which the reactive-bond loop has been inserted into the A sheet. Loop insertion thus leads to exposure of new epitopes in antithrombin that are not present in the intact inhibitor. Notably, the specific antibodies also reacted with antithrombin−proteinase complexes in the same manner as with the loop-inserted forms of antithrombin. This finding infers that the reactive-bond loop is inserted in the A β-sheet also in the proteinase complexes.

In this work, we have identified a linear epitope in bovine antithrombin that reacts with antibodies specific for reactive-bond-cleaved antithrombin or the antithrombin−thrombin complex. This epitope is a hexapeptide sequence comprising residues 342−347, Glu-Asp-Leu-Phe-Ser-Pro. In the structure of cleaved bovine antithrombin (Mourey et al., 1993), this sequence is located on the surface of the protein just carboxy-terminal of helix I. The Phe residue of this sequence is highly conserved among serpins and interacts with the protein core in the structures of antithrombin and other serpins. The epitope region presumably becomes accessible to antibodies as a result of the Phe residue transmitting the conformational change induced by insertion of the reactive-bond loop into the A β-sheet to this region.

MATERIALS AND METHODS

Intact and reactive-bond-cleaved bovine antithrombin were purified as described previously (Nordenman et al., 1977; Björk et al., 1993). Concentrations of the proteins were determined from absorbance measurements (Nordenman et al., 1977). Reactive-bond-cleaved antithrombin was labeled with ^{125}I by the Iodo-Gen procedure (Pierce, Rockford, IL; Caix et al., 1987) to a specific activity of 4.5×10^4 Bq/μg.

Two antisera against reactive-bond-cleaved bovine antithrombin and one against the bovine antithrombin−thrombin complex, all raised in rabbits (Wallgren et al., 1981), were used. The IgG[1] fraction was isolated from these antisera by ion exchange chromatography on DEAE-cellulose (Björk & Tanford, 1971). Antibodies reacting with intact bovine antithrombin were removed by passing ~7 mg of IgG over three consecutive 1 mL columns of Affi-Gel 15 (Bio-Rad, Hercules, CA), to which had been linked ~4 mg of antithrombin per milliliter of gel.

The absence of reactivity of the antibody preparations with intact bovine antithrombin, as well as their specificity for forms of the inhibitor having the reactive bond inserted in the A β-sheet and for complexes with proteinases, was checked by immunodiffusion as described previously (Björk et al., 1993). The lack of reactivity with intact antithrombin was also ascertained by experiments in which intact or cleaved antithrombin was allowed to compete with radiolabeled cleaved inhibitor for binding to the antibodies. ^{125}I-labeled cleaved antithrombin (0.25 μg) and increasing amounts of unlabeled intact or cleaved antithrombin were incubated with antibody (1.5 and 50 μg of IgG for the antibodies against cleaved antithrombin and the antithrombin−thrombin complex, respectively) in a total volume of 200 μL for 2 h at room temperature. A suspension of fixed *Staphylococcus aureus* cells was then added to bind the antibodies; the cells were washed, and the amount of labeled cleaved antithrombin bound to the antibodies was determined by scintillation counting. Further details were as in Harlow and Lane (1988).

Intact and reactive-bond-cleaved antithrombin were denatured and reduced and alkylated as described previously (Björk & Jörnvall, 1986). The proteins (1.0−1.3 mg) were subsequently cleaved with cyanogen bromide (1.7 mg) in 0.3 mL of 70% (v/v) formic acid for 24 h at 25 °C and were then lyophilized after 10-fold dilutions with water.

SDS−PAGE was done with the tricine buffer system on a 16.5% T, 2.6% C separating gel, overlaid with a 10% T, 2.6% C spacer gel and a 4% T, 2.6% C stacking gel (Schägger & von Jagov, 1987). The gels were stained with Coomassie Brilliant Blue R250 or electroblotted to a poly(vinylidene difluoride) membrane (Qiabrane PVDF; Qiagen, Chatsworth, CA). For detection of proteins or peptides binding the specific antibodies against reactive-bond-cleaved antithrombin, the membrane was treated with glutaraldehyde (Gershoni & Palade, 1983) and then allowed to react for 1 h with the antibodies at a concentration of ~1 μg of IgG/mL. The binding of the primary antibodies was detected with horseradish peroxidase-conjugated anti-rabbit IgG in conjunction with the enhanced chemiluminescence (ECL; Amersham, Little Chalfont, U.K.) system. For analyses of N-terminal sequences, the membrane was stained with Coomassie Brilliant Blue R250 and appropriate bands were excised and the peptides sequenced directly in an Applied Biosystems (Foster City, CA) 470A gas phase sequencer, connected on-line to a 120A PTH analyzer.

Solid phase synthesis of peptides was done on a derivatized cellulose membrane (Blankemeyer-Menge et al., 1990) with the SPOTs system (Cambridge Research Biochemicals, Northwich, U.K.). The membrane was probed with the specific antibodies against reactive-bond-cleaved antithrombin and the antithrombin−thrombin complex at concentrations of ~8 and ~35 μg of IgG/mL, respectively. Binding of the antibodies against reactive-bond-cleaved antithrombin to the peptides was detected with a β-galactosidase-conjugated secondary anti-rabbit IgG antibody and the substrate, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, yielding a blue product on cleavage. The details of the procedures were as described by the manufacturer. Binding of the antibodies against the antithrombin−thrombin complex was detected by horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence.

RESULTS

In this work, IgG fractions containing antibodies against bovine reactive-bond-cleaved antithrombin or antithrombin in complex with thrombin were made specific for these forms of the inhibitor by three consecutive rounds of affinity chromatography on matrix-linked intact bovine antithrombin. No detectable amounts of protein were adsorbed during the last two passages. Like the absorbed antisera used in previous studies (Björk et al., 1993), the antibodies prepared in this way did not react with the intact inhibitor in immunodiffusion analyses (not shown). Moreover, unlabeled intact antithrombin did not compete with radioactively labeled reactive-bond-cleaved antithrombin for binding to any of the three antibody preparations, as shown for one preparation against the reactive-bond-cleaved inhibitor (Figure 1). In contrast, unlabeled cleaved antithrombin competed efficiently (Figure 1). In both types of analysis, the same results were

[1] Abbreviations: IgG, immunoglobulin G; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; tricine, *N*-tris(hydroxymethyl)methylglycine.

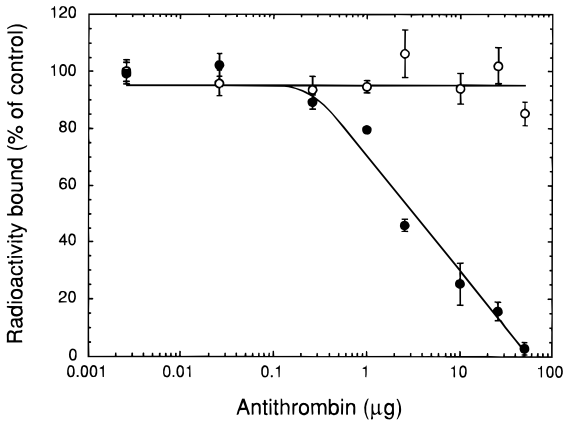

FIGURE 1: Competition between intact or reactive-bond-cleaved bovine antithrombin and ^{125}I-labeled reactive-bond-cleaved antithrombin for binding to specific antibodies against the cleaved inhibitor: (○) intact antithrombin and (●) cleaved antithrombin. The amount of radioactivity bound is expressed in percent of that of a control without unlabeled antithrombin. The values are averages of six analyses; the vertical bars represent standard errors.

obtained also after the IgG fractions had been subjected to only two rounds of affinity chromatography. No detectable amounts of antibodies against intact antithrombin thus remained in the final preparations. The antibodies had the same specificity for reactive-bond-cleaved antithrombin and other forms of the inhibitor in which the reactive-bond loop had been inserted into the A β-sheet, and for antithrombin−proteinase complexes, as the corresponding absorbed antisera used previously (Björk et al., 1993).

Immunoblotting showed that the specific antibodies against reactive-bond-cleaved antithrombin, which did not react with the nondenatured intact inhibitor, bound to both intact and cleaved antithrombin after SDS−PAGE (not shown). At least one of the specific epitopes in the reactive-bond-cleaved inhibitor thus must be a linear epitope, hidden in intact antithrombin but becoming accessible on denaturation of the intact inhibitor, as well as on cleavage of the reactive bond.

SDS−PAGE of CNBr-degraded intact antithrombin, followed by immunoblotting, showed two bands that reacted with the specific antibodies against reactive-bond-cleaved antithrombin (Figure 2). The N-terminal sequence of the largest, but weakly stained, peptide was Pro-Arg-Phe-Arg-Ile-Glu whereas that of the smaller, more intensely stained, peptide was Gly-Leu-Glu-Asp-Leu-Phe. Comparison with the sequence of bovine antithrombin (Mejdoub et al., 1991) indicated that the smaller peptide was the CNBr fragment 340−424 and that the larger peptide comprised residues 322−424 and thus was the result of incomplete cleavage after Met-339. However, it cannot be excluded that one or both fragments extended to the carboxy terminus of the protein, Asp-433, as a result of incomplete cleavage also at Met-424.

Similar analysis of CNBr-degraded reactive-bond-cleaved antithrombin showed one dominating antibody-reacting peptide, which was smaller than either of the two peptides from the intact inhibitor (Figure 2). Its N-terminal sequence was Gly-Leu-Glu-Asp-Leu-Phe, demonstrating that it was derived from cleavage after Met-339, like the smaller peptide from intact antithrombin. This finding, together with the size of the peptide and the fact that it was obtained from reactive-bond-cleaved antithrombin, indicated that it extended from Gly-340 to Arg-394 of the cleaved reactive bond. At least one neoepitope should thus be located in this segment of

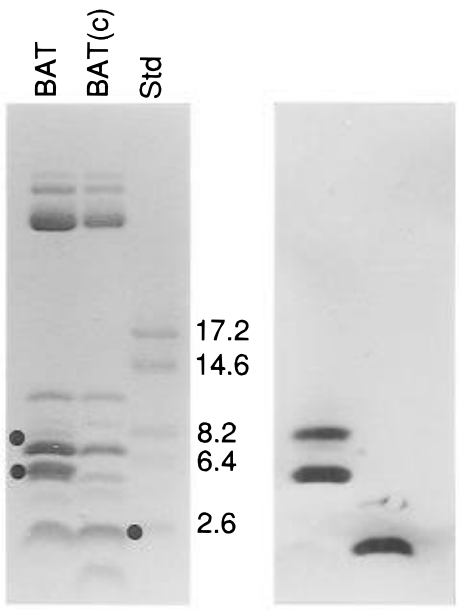

FIGURE 2: SDS−PAGE and immunoblotting of fragments obtained by CNBr degradation of intact and reactive-bond-cleaved bovine antithrombin: left, stained gel; and right, immunoblot. BAT is intact antithrombin, and BAT (c) is cleaved antithrombin. The immunoblot was developed with specific antibodies against reactive-bond-cleaved antithrombin. The bands in the stained gel corresponding to the antibody-binding bands in the immunoblot are marked with a black dot. Std is standards with the molecular masses in kilodaltons indicated.

the antithrombin polypeptide chain, regardless of the uncertainty of the extension of the two antibody-binding peptides from intact antithrombin.

The epitope or epitopes were identified by analyses of the binding of the specific antibodies to synthetic peptides. A series of dodecapeptides covering the region between Phe-330 and the carboxy terminus, Asp-433, each peptide being offset by one residue, was synthesized on a cellulose membrane. Both preparations of specific antibodies against reactive-bond-cleaved antithrombin bound to peptides containing the hexapeptide sequence Glu-Asp-Leu-Phe-Ser-Pro (Figure 3A), but to no other peptide synthesized (not shown). This sequence corresponds to residues 342−347 of bovine antithrombin (Mejdoub et al., 1991). The specific antibodies against the antithrombin−thrombin complex also bound to peptides containing this sequence (not shown).

The epitope was further characterized by probing the binding of the antibodies against reactive-bond-cleaved antithrombin to a series of decapeptides containing the hexapeptide sequence identified, but with each peptide having one of the six residues substituted by alanine. Replacement of Asp-343, Phe-345, and Pro-347 with alanine eliminated the binding (Figure 3B), indicating that these residues are essential for interaction with the antibodies. In contrast, replacement of Glu-342, Leu-344, and Ser-346 had no apparent effect on antibody binding, consistent with these residues contributing little to the interaction. In keeping with these results, the antibodies also bound to a peptide containing the corresponding amino acid sequence in human antithrombin, Val-Asp-Leu-Phe-Ser-Pro (not shown), in which Glu-342, of little importance for the interaction, is replaced by Val (Petersen et al., 1979; Bock et al., 1982).

DISCUSSION

In this work, we have identified a linear epitope in bovine antithrombin that reacts with antibodies specific for anti-

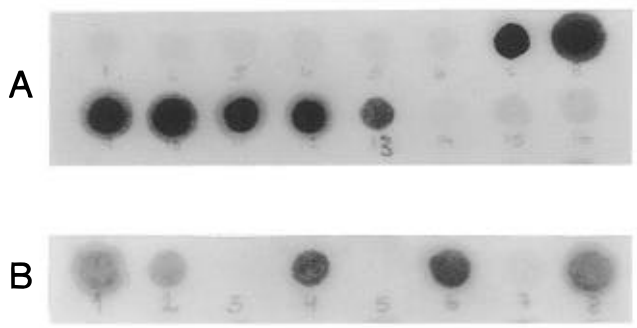

FIGURE 3: Binding of specific antibodies against reactive-bond-cleaved bovine antithrombin to peptides synthesized on a cellulose membrane. (A) Dodecapeptides covering the region between Phe-330 and Val-356 of bovine antithrombin with the following sequences: spot 1, FSVKEQLQDMGL; spot 2, SVKEQLQDMGLE; spot 3, VKEQLQDMGLED; spot 4, KEQLQDMGLEDL; spot 5, EQLQDMGLEDLF; spot 6, QLQDMGLEDLFS; spot 7, LQDMGLEDLFSP; spot 8, QDMGLEDLFSPE; spot 9, DMGLEDLFSPEK; spot 10, MGLEDLFSPEKS; spot 11, GLEDLFSPEKSR; spot 12, LEDLFSPEKSRL; spot 13, EDLFSPEKSRLP; spot 14, DLFSPEKSRLPG; spot 15, LFSPEKSRLPGI; and spot 16, FSPEKSRLPGIV. The sequence common to all antibody-binding peptides, EDLFSP, is underlined. No binding to other peptides in the region from Phe-330 to the carboxy terminus, Asp-433, was detected. (B) Decapeptides containing the deduced epitope from Glu-342 to Pro-347 (underlined), but with each of the six residues of the epitope replaced by Ala. The sequences were as follows: spot 1, GLEDLFSPEK (control); spot 2, GLADLFSPEK; spot 3, GLEALFSPEK; spot 4, GLEDAFSPEK; spot 5, GLEDLASPEK; spot 6, GLEDLFAPEK; spot 7, GLEDLFSAEK; and spot 8, GLEDLFSPEK (control).

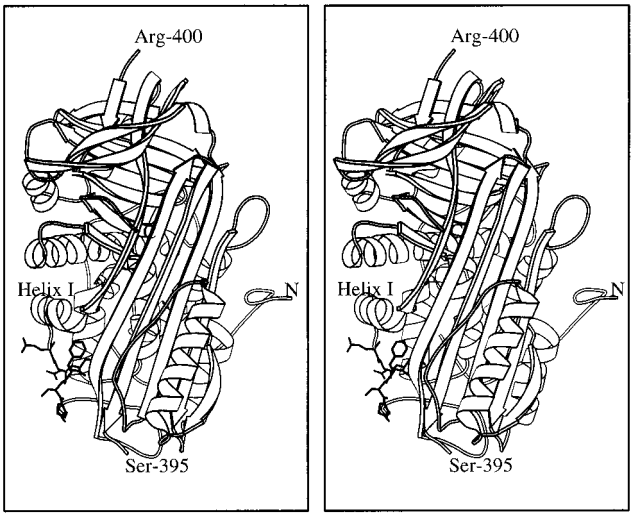

FIGURE 4: Location in the three-dimensional structure of cleaved bovine antithrombin of the epitope appearing on insertion of the reactive-bond loop into the A β-sheet. The main body of the protein is represented by a ribbon diagram of the polypeptide backbone, whereas the amino acids of the epitope are shown with their side chains. The cleaved bond is Ser-395−Leu-396, one bond carboxy-terminal of the reactive bond. The carboxy-terminal segment of the cleaved reactive-bond loop is at the top of the molecule (although no electron density is apparent between residues 396 and 399), whereas the amino-terminal segment of the loop is inserted in the A β-sheet. Helix F with the underlying A β-sheet is at the front and right of the molecule. The figure was drawn on the basis of the structure determined by Mourey et al. (1993) with Bobscript v. 2.0 (Robert Esnouf's extensions to Molscript v. 1.4; Kraulis, 1991).

thrombin forms in which the reactive-bond loop has been inserted as a middle strand in the main β-sheet, the A sheet. These antibodies also react with antithrombin−proteinase complexes, suggesting that the loop is similarly inserted in such complexes. The epitope is located at the surface of cleaved bovine antithrombin in the one-turn helix I1 on the carboxy-terminal side of helix I (Figure 4; Huber & Carrell, 1989; Mourey et al., 1993). The corresponding, highly similar, epitope in the human inhibitor also bound the antibodies, in keeping with the previously demonstrated cross-reactivity between loop-inserted forms of bovine and human antithrombin (Björk et al., 1993). Of the six amino acid residues of the epitope, only three, Asp-343, Phe-345, and Pro-347, were crucial for the interaction.

Comparisons between the X-ray structures of intact and latent (i.e. loop-inserted) human antithrombin or between intact human and cleaved bovine antithrombin (Mourey et al., 1993; Schreuder et al., 1994; Carrell et al., 1994) revealed no major differences in the three-dimensional arrangement of the residues comprising the epitope identified. This observation is in contrast to what might have been expected, as the epitope is only accessible to the antibodies in cleaved antithrombin and other loop-inserted antithrombin forms. However, the reactive-bond loop is partly inserted into the A sheet in the crystal forms of intact antithrombin for which the X-ray structures were determined (Schreuder et al., 1994; Carrell et al., 1994), and the conformational change leading to exposure of the epitope therefore may have occurred in these forms. This possibility is supported by the corresponding region in the crystal structure of intact α_1-antichymotrypsin, in which the loop is not inserted, having a notably different conformation than in the cleaved inhibitor (Wei et al., 1994; Baumann et al., 1991; S. Bock, personal communication). The finding that the epitope is not accessible in intact antithrombin is thus consistent with the reactive-bond loop being fully exposed in the intact inhibitor, as in α_1-antichymotrypsin and α_1-proteinase inhibitor. A further possibility is that loop insertion may weaken the intramolecular interactions in the epitope region, without appreciably affecting the structure of this region, thereby allowing the antibodies to induce a conformational change required for tight binding. That such an induced fit is necessary is suggested by the observation that Phe-345, an essential binding residue, is oriented toward the interior in the protein (Figure 4; Mourey et al., 1993; Schreuder et al., 1994; Carrell et al., 1994) and therefore presumably has to move appreciably to interact with the antibodies.

The essential Phe-345 residue of the observed epitope is highly conserved in members of the serpin superfamily (Huber & Carrell, 1989; Janssen et al., 1995). The preceding residue is usually hydrophobic, whereas the other residues of the epitope are more variable. The Phe residue has been shown to tie the helix I1 region to the protein core in the structure of cleaved α_1-proteinase inhibitor (Huber & Carrell, 1989) and appears to have a similar role in the antithrombin and cleaved α_1-antichymotrypsin structures (Baumann et al., 1991; Mourey et al., 1993; Schreuder et al., 1994; Carrell et al., 1994). The conserved Phe residue may be of importance for the stability of serpins, as indicated by the properties of a congenital variant of the noninhibitory serpin, thyroxine-binding globulin (Janssen et al., 1995). This protein normally has a Tyr residue in the position of the conserved Phe in most other serpins. Replacement of this Tyr residue with a Phe in the mutant serpin appreciably increases the thermal stability of the protein, consistent with more favorable hydrophobic interactions with the main body of the protein. These observations indicate that the conformational change induced by expansion of the A β-sheet following insertion

of the reactive-bond loop is transmitted to the epitope region in antithrombin through Phe-345. Moreover, the conservation of this residue among serpins and its apparent interaction with the protein core in several serpins suggest that structural changes akin to those detected by the specific antibodies in this work may be a general sequel to loop insertion in serpins.

As our antibodies specific for the loop-inserted forms of antithrombin give precipitin lines in immunodiffusion analyses, they must also recognize other epitopes in these forms of the inhibitor. Such additional epitopes were not identified by the methods used. However, linear epitopes involving a Met residue would have escaped detection, as the initial localization of antibody-binding regions in the antithrombin sequence involved cyanogen bromide cleavage. Moreover, most or all of the additional epitopes most likely are dependent on the conformation of the protein. Recent studies of two monoclonal antibodies, specific for cleaved and complex-bound C1-inhibitor or plasminogen activator inhibitor-1, indicated that these antibodies bind to such conformation-dependent epitopes in the region around strand 1C (Eldering et al., 1995; Debrock & Declerck, 1995). One or more of the putative conformation-dependent epitopes in loop-inserted forms of antithrombin recognized by the specific antibodies in our work may be similarly located in the corresponding region of antithrombin.

Asakura et al. (1988, 1989, 1990) have produced a monoclonal antibody against the human antithrombin−thrombin complex that bound only weakly to intact antithrombin. It reacted also with reactive-bond-cleaved antithrombin and may have had similar specificity for loop-inserted antithrombin forms as the antibodies in this work. The epitope of the monoclonal antibody was shown to be residues 382−386, Ala-Ala-Ala-Ser-Thr, i.e. the P12−P8 residues of the reactive-bond loop. The antibody promoted the formation of cleaved antithrombin by the substrate pathway during the reaction of antithrombin with thrombin (Olson & Björk, 1994), presumably by binding to an intermediate of the reaction. In spite of the sequence of the proposed epitope being identical in bovine antithrombin, no binding of the specific antibodies against loop-inserted forms of the bovine inhibitor to peptides containing this sequence could be detected in this work.

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