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Immunologic Evidence for Insertion of the Reactive-Bond Loop of Antithrombin into the A β-Sheet of the Inhibitor during Trapping of Target Proteinases†

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ABSTRACT: Identical or highly similar antigenic determinants, not present in the intact inhibitor, were induced in antithrombin on cleavage of the reactive bond, on formation of a complex between antithrombin and a synthetic reactive-loop tetradecapeptide, and on partial denaturation of antithrombin at low concentrations of guanidinium chloride. Previous studies indicate that the common structural feature of these three modified forms of antithrombin is that the region of the reactive-bond loop on the amino-terminal side of the reactive bond, or the corresponding synthetic peptide, is inserted as a middle strand in the main β-sheet of the inhibitor, the A sheet. The new epitopes in the three modified antithrombin forms therefore most likely are exposed as a result of this insertion. Identical or highly similar epitopes were exposed also in complexes between antithrombin and thrombin or factor Xa, strongly suggesting that a substantial segment of the reactive-bond loop is inserted into the A sheet also in these complexes. In contrast, the new epitopes were not exposed in antithrombin on binding of heparin, implying that the conformational change induced by heparin does not involve such loop insertion. These results provide the first experimental verification of recent hypotheses that insertion of the reactive-bond loop of serpins into the A β-sheet is involved in the binding of target proteinases.

Antithrombin, the major plasma inhibitor of coagulation proteinases [for reviews, see Björk et al. (1989) and Olson and Björk (1992)], is a member of the serpin superfamily of proteins (Carrell & Travis, 1985). Besides antithrombin, this family also contains most other plasma serine proteinase inhibitors, e.g., α_1-proteinase inhibitor, α_1-antichymotrypsin, α_2-antiplasmin, C1-inhibitor, and plasminogen activator inhibitor-1, as well as several noninhibitory proteins, such as ovalbumin and angiotensinogen (Huber & Carrell, 1989). The inactivation of a target proteinase by an inhibitory serpin is initiated by the proteinase interacting with a specific reactive bond of the inhibitor and is effected by a subsequent trapping mechanism associated with a conformational change of the inhibitor. This trapping leads to the formation of a kinetically stable, possibly covalently linked, serpin–proteinase complex that slowly dissociates to inactive, reactive-bond-cleaved inhibitor and free enzyme (Travis & Salvesen, 1983; Carrell & Boswell, 1986; Huber & Carrell, 1989; Bode & Huber, 1992; Olson & Björk, 1992). The mechanism of action of serpins thus differs substantially from that of low-molecular-weight protein inhibitors of serine proteinases (Laskowski & Kato, 1980; Bode & Huber, 1992).

The nature of the trapping reaction has been the subject of appreciable speculation but is still unknown. No X-ray structure of an active serpin has been reported, although the structures of several inactive inhibitors, which either are cleaved at or near the reactive bond (α_1-proteinase inhibitor, antithrombin, and α_1-antichymotrypsin) or are in a latent conformation (plasminogen activator inhibitor-1), have been solved (Loebermann et al., 1984; Mourey et al., 1990; Baumann et al., 1991; Mottonen et al., 1992). The reactive-bond regions of the active inhibitors must differ substantially from the corresponding regions of these structures. The two residues of the cleaved bond in the proteolytically modified inhibitors are thus at opposite ends of the molecule, and the segment amino-terminal of this bond constitutes a central strand of the major β-sheet of the cleaved inhibitor, the six-stranded A sheet (Figure 1). In the latent plasminogen activator inhibitor-1 the corresponding region of the chain occupies the same position, rendering the reactive bond inaccessible, although the polypeptide chain is intact. Considerable evidence indicates that this segment of the active inhibitors instead forms part of an exposed reactive-bond loop analogous to that formed by the corresponding segment of the noninhibitory serpin, ovalbumin, resulting in a five-stranded A sheet (Figure 1; Huber & Carrell, 1989; Engh et al., 1990; Stein et al., 1990; Baumann et al., 1991; Bode & Huber, 1992). This putative structure, together with additional circumstantial evidence, has led to the proposal of two partly different models for the mechanism of action of serpins. One of these, which may be termed the induced conformational change model, suggests that the trapping of a target proteinase occurs by the enzyme inducing the region of the reactive-bond loop on the amino-terminal side of the reactive bond to insert into the A sheet (Skriver et al., 1991; Björk et al., 1992b). An alternative proposal, the preequilibrium conformational change model, is that the inhibitor exists in an equilibrium between two conformational states, one inactive state with the reactive-bond loop fully exposed and one active state with the loop partially inserted into the A sheet. The proteinase

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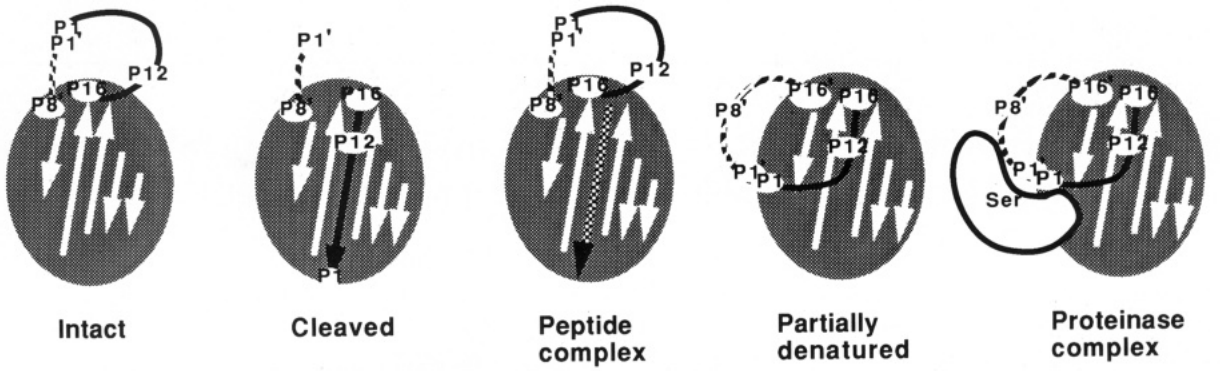

FIGURE 1: Schematic representation of the experimentally determined X-ray structure of reactive-bond-cleaved antithrombin and of the deduced structures of intact antithrombin, a complex between intact antithrombin and a reactive-loop tetradecapeptide, partially denatured antithrombin, and a complex between antithrombin and a target proteinase. White arrows indicate strands of the A β-sheet of the inhibitor. The solid and broken black lines denote the amino-terminal and carboxy-terminal parts, respectively, of the reactive-bond loop. The synthetic tetradecapeptide is crosshatched. The positions of pertinent residues are indicated by numbering from the reactive bond according to the convention introduced by Schechter and Berger (1967).

is assumed to bind only to the latter state, in which the loop has a shape complementary to the active-site cleft of the enzyme, thereby shifting the equilibrium to this state (Carrell et al., 1991; Björk et al., 1992b). Both these models propose that partial insertion of the reactive-bond loop into the A sheet is an essential feature of complex formation between serpins and target proteinases (Figure 1). However, that such insertion indeed occurs has not yet been demonstrated.

In this paper, we present the first experimental evidence that the reactive-bond loop of antithrombin is inserted into the A β-sheet of the inhibitor in complexes between this serpin and two of its target proteinases. Previous studies have shown that binding of thrombin or factor Xa by antithrombin leads to exposure of new epitopes in the inhibitor that are not present in uncomplexed antithrombin (Collen & de Cock, 1978; Lau & Rosenberg, 1980; Wallgren et al., 1981; Asakura et al., 1990). We now show that identical epitopes are exposed also in reactive-bond-cleaved antithrombin, in a complex between a synthetic reactive-loop peptide and antithrombin, and in antithrombin partially denatured at low concentrations of guanidinium chloride. As previous studies indicate that the common feature of these three forms of antithrombin is that part of the reactive-bond loop, or the corresponding synthetic peptide, is inserted as a middle strand in the A β-sheet (Figure 1), the new epitopes most likely are exposed as a result of this insertion. The exposure of the same epitopes in antithrombin–proteinase complexes indicates that such insertion has occurred also in the complexes.

MATERIALS AND METHODS

Bovine and human antithrombin were purified and their concentrations determined as described previously (Nordenman et al., 1977). Reactive-bond-cleaved antithrombins were produced by incubating the inhibitors (0.5 mg/mL) with bovine thrombin (40 μg/mL) and heparin with high affinity for antithrombin (14 μg/mL) for 30 min at 25 °C in 8 mM sodium phosphate, pH 7.4, ionic strength 0.03 (Olson, 1985). The cleaved inhibitors were purified by affinity chromatography on heparin–agarose (Björk & Fish, 1982a). Partially denatured, inactive bovine antithrombin was obtained by treating antithrombin (~10 mg/mL) with 0.9 M guanidinium chloride in 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4, for 10 h at 4 °C, followed by overnight dialysis against the phosphate buffer alone (Fish et al., 1985; Carrell et al., 1991).

An N-acetylated tetradecapeptide having the sequence of residues 380–393, i.e., the P_1–P_{14} segment, of human antithrombin (Ser-Glu-Ala-Ala-Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-Gly-Arg) was synthesized by the solid-phase procedure (Björk et al., 1992a). Complexes between bovine or human antithrombin and this peptide were prepared by incubating antithrombin (2 mg/mL) with the peptide (1 mg/mL) for 24 h at 37 °C in 1 M Tris-HCl and 0.5 M formate, pH 7.4, and isolating the complexes by heparin affinity chromatography (Björk et al., 1992a). The bovine antithrombin–peptide complex had properties identical to those of the complex of human antithrombin with the peptide, indicating that the tetradecapeptide was bound to bovine antithrombin as a middle strand of the main β-sheet of the inhibitor in a manner similar to that of human antithrombin (Björk et al., 1992a).

Bovine α-thrombin, ~85% active by active-site titration with 4-nitrophenyl 4-guanidinobenzoate (Chase & Shaw, 1970), bovine factor X, and the factor X-activating enzyme from Russell's viper venom were gifts from Drs. Johan Stenflo and Egon Persson, University of Lund, Lund, Sweden. Bovine factor X (1 mg/mL) was activated to factor Xa with the activating enzyme (2 μg/mL) at pH 7.4 for 30 min at 37 °C in the presence of 10 mM $CaCl_2$. Human α-thrombin, ~90% active, was a gift from Dr. John Fenton, New York State Department of Public Health, Albany, NY. Human factor Xa, ~90% active, was purified as described previously (Björk et al., 1992b). Concentrations of the proteins were determined from absorbance measurements (Björk & Fish, 1982b; Björk et al., 1982; Olson et al. 1992). Complexes of bovine antithrombin with bovine thrombin or factor Xa and of human antithrombin with human thrombin or factor Xa were prepared by incubating the inhibitors (1.0–1.5 mg/mL) with the enzymes at a molar ratio of inhibitor to enzyme of 1.1 to 1 in 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4, for 2 h (for thrombin) or 4 h (for factor Xa) at 25 °C. More than 98% of both enzymes formed a complex with antithrombin under these conditions.

Commercial heparin from porcine mucosa (150 IU/mg) was obtained from Kabi-Pharmacia, Stockholm, Sweden. Heparin with high affinity for antithrombin was isolated by affinity chromatography on immobilized antithrombin (Höök et al., 1976; Nordenman & Björk, 1978).

Immunodiffusion (Ouchterlony, 1958) was done in 1% agarose gels in 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4. In some experiments, commercial heparin (1 mg/mL) was included in the gels. Antisera against bovine reactive-bond-cleaved antithrombin and the bovine antithrombin–thrombin complex were raised in rabbits and absorbed with

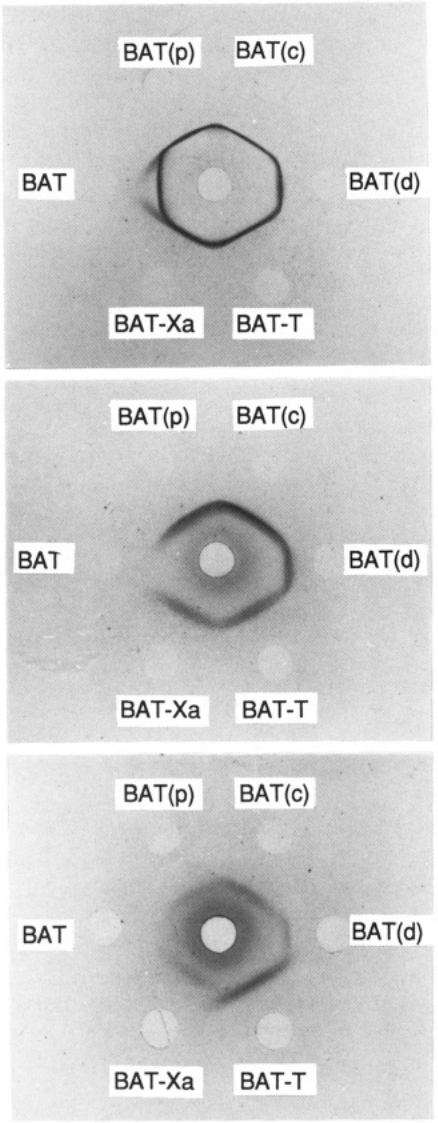

FIGURE 2: Immunodiffusion analyses of intact bovine antithrombin, differently modified forms of bovine antithrombin, and bovine antithrombin–proteinase complexes. (A, top) An antiserum against reactive-bond-cleaved bovine antithrombin; (B, middle) the same antiserum absorbed with intact bovine antithrombin; (C, bottom) an antiserum against the bovine antithrombin–thrombin complex, absorbed with intact bovine antithrombin. Abbreviations: BAT, intact bovine antithrombin; BAT(p), complex of bovine antithrombin with a synthetic reactive-loop tetradecapeptide; BAT(c), reactive-bond-cleaved bovine antithrombin; BAT(d), bovine antithrombin partially denatured at low concentrations of guanidinium chloride; BAT-T, bovine antithrombin–thrombin complex; BAT-Xa, bovine antithrombin–factor Xa complex. Antithrombin concentrations were 1.0 mg/mL in (A), 0.5 mg/mL in (B), and 0.3–0.5 mg/mL in (C).

intact antithrombin as described previously (Wallgren et al., 1981).

RESULTS

Different forms of bovine antithrombin were analyzed by immunodiffusion with an antiserum against the reactive-bond-cleaved bovine inhibitor. These experiments showed complete immunological identity between the cleaved inhibitor, a complex of a synthetic reactive-loop tetradecapeptide with the inhibitor, antithrombin partially denatured at low concentrations of guanidinium chloride, and complexes of antithrombin with thrombin or factor Xa (Figure 2A). In contrast, the intact, uncomplexed inhibitor showed only partial identity with these forms of antithrombin. Such partial identity

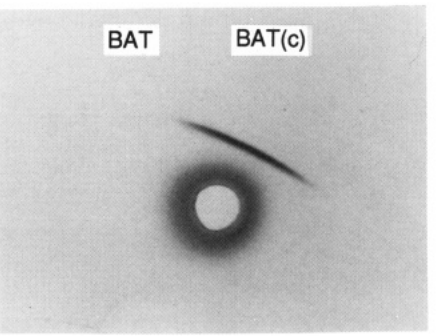

FIGURE 3: Immunodiffusion analyses of intact and reactive-bond-cleaved bovine antithrombin in the presence of heparin. The antiserum was against reactive-bond-cleaved bovine antithrombin and was absorbed with the intact inhibitor. Abbrevations: BAT, intact bovine antithrombin; BAT(c), reactive-bond-cleaved bovine antithrombin. The protein solutions and the gel contained 1 mg/mL unfractionated heparin, i.e., ~0.35 mg/mL heparin with high affinity for antithrombin, giving >99% saturation of the intact inhibitor (Olson & Björk, 1992). Antithrombin concentrations were 0.5 mg/mL.

between intact antithrombin and the cleaved inhibitor or the antithrombin–proteinase complexes is in agreement with earlier work (Wallgren et al., 1981).

Absorption of the antiserum against reactive-bond-cleaved bovine antithrombin with intact bovine antithrombin removed all reactivity against the intact inhibitor (Figure 2B). The absorbed antiserum showed complete identity between the cleaved inhibitor, the antithrombin–tetradecapeptide complex, the partially denatured antithrombin, and the two antithrombin–proteinase complexes. No reactivity between the absorbed antiserum and intact antithrombin was induced when the immunodiffusion analyses were done in the presence of heparin at a concentration sufficient to saturate the inhibitor (Figure 3).

An antiserum against the bovine antithrombin–thrombin complex absorbed with intact antithrombin gave results similar to those of the corresponding absorbed antiserum against the cleaved inhibitor (Figure 2C). This antiserum thus gave precipitin lines with the three differently modified forms of uncomplexed antithrombin, as well as with the two antithrombin–proteinase complexes, but not with the intact inhibitor. In addition, the antiserum showed some reactivity against the antithrombin–thrombin complex not given by the antithrombin–factor Xa complex or the other antithrombin forms, as evident by weak spurs over the neighboring precipitin lines. This behavior presumably is due to epitopes in thrombin. Although the antiserum was raised against the antithrombin–thrombin complex, the possibility cannot be excluded that it may have been directed, at least partly, against free reactive-bond-cleaved antithrombin and thrombin, formed by the complex having dissociated during the immunization period. In such a case, the antiserum would be analogous to that against reactive-bond-cleaved antithrombin (Figure 2B).

Previous studies with antisera against intact human or bovine antithrombin failed to detect an immunologic cross-reactivity between the two proteins (Nordenman et al., 1977). However, in this work intact human antithrombin was found to react weakly with the antiserum against the reactive-bond-cleaved bovine inhibitor (Figure 4). Conversion of human antithrombin to the reactive-bond-cleaved form or production of complexes between human antithrombin and the synthetic tetradecapeptide, thrombin, or factor Xa induced additional epitopes in the inhibitor that reacted with the antiserum against reactive-bond-cleaved bovine antithrombin (Figure 4).

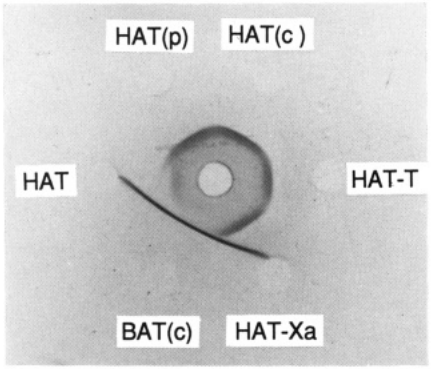

FIGURE 4: Immunodiffusion analyses of intact human antithrombin, differently modified forms of human antithrombin, and human antithrombin–proteinase complexes. The antiserum was against reactive-bond-cleaved bovine antithrombin, showing only weak reactivity with the intact human inhibitor. Abbreviations: HAT, intact human antithrombin; HAT(p), complex of human antithrombin with a synthetic reactive-loop tetradecapeptide; HAT(c) reactive-bond-cleaved human antithrombin; HAT-T, human antithrombin–thrombin complex; HAT-Xa, human antithrombin–factor Xa complex; BAT(c), reactive-bond-cleaved bovine antithrombin. Human and bovine antithrombin concentrations were 0.5 and 1.0 mg/mL, respectively.

DISCUSSION

These results show that new, identical or highly similar antigenic determinants are induced in antithrombin on cleavage of the reactive bond, on formation of a complex between the inhibitor and a synthetic-loop tetradecapeptide, and on partial denaturation of the inhibitor at low concentrations of guanidinium chloride. Previous studies indicate that the common structural change that has occurred in these three modified forms of antithrombin is that the region of the reactive-bond loop on the amino-terminal side of the reactive bond, or the corresponding synthetic peptide, has been inserted as a middle strand in the main β-sheet of the inhibitor, the A sheet (Figure 1; Engh et al., 1990; Stein et al., 1990; Mourey et al., 1990; Schultze et al., 1990; Carrell et al., 1991; Björk et al., 1992a,b; Mast et al., 1992). The new epitopes in the modified antithrombin forms thus most likely are internally located or otherwise masked in intact antithrombin and are exposed as a result of this insertion. The finding that identical or highly similar epitopes are exposed also in complexes between antithrombin and two of its target proteinases strongly suggests that a substantial segment of the reactive-bond loop has been inserted into the A sheet also in these complexes (Figure 1). Interestingly, common or related epitopes appear to be induced in human and bovine antithrombin on such loop insertion, in spite of at most a weak cross-reactivity between the intact inhibitors from the two species. The exposed epitopes thus are highly conserved between humans and cows.

This work provides the first experimental verification of recent hypotheses that insertion of the reactive-bond loop into the A β-sheet of serpins is an essential feature of the mechanism of inactivation of proteinases (Skriver et al., 1991; Carrell et al., 1991). The results are compatible with both the induced conformational change and preequilibrium conformational change models proposed for such insertion (Skriver et al., 1991; Carrell et al. 1991; Björk et al., 1992b), as both models would lead to the same final state of serpin–proteinase complexes with the loop inserted into the A sheet, in agreement with our findings. In the case of the preequilibrium conformational change model, however, it is apparent that insertion of the loop in intact serpins to the same extent as in complexes with proteinases must be assumed to occur in at most a small proportion of the molecules, as the epitopes indicative of such insertion were not shown by intact antithrombin. Nevertheless, it is possible that in both models one or two residues of the loop may be inserted in the intact inhibitor (Mast et al., 1992; Schulze et al., 1992) without inducing the exposure of these epitopes.

The immunodiffusion analyses in the presence of heparin indicate that activation of antithrombin by heparin does not involve insertion of the reactive-bond loop of the inhibitor into the A sheet to an extent detectable with the immunological method used. This observation argues against a proposed mechanism of heparin activation involving such loop insertion (Carrell et al., 1991). In this mechanism, heparin is postulated to induce a similar partial insertion of the loop into the A sheet as that assumed to enable the loop to bind target proteinases in the preequilibrium conformational change model, i.e., leading to a conformation of the loop complementary to the active-site region of the proteinases. Instead, our results are consistent with heparin inducing a conformational change in the reactive-bond loop that activates the inhibitor by a mechanism independent of that involved in the trapping of a proteinase in a stable complex.

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