# THE SITE IN HUMAN ANTITHROMBIN FOR FUNCTIONAL PROTEOLYTIC CLEAVAGE BY HUMAN THROMBIN

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## 1. Introduction

Antithrombin neutralization of thrombin activity involves the formation of a highly stable enzyme–inhibitor complex [1,2]. The rate of this inactivation is markedly enhanced by the presence of heparin [2]. Appreciable amounts of antithrombin are also converted by thrombin to a proteolytically modified, non-complexed product when the two proteins react in near-equimolar ratios [3]. The generation of this modified antithrombin form is increased in the presence of heparin (I. B. and W. W. Fish, unpublished; K. K. Lavine and C. M. Jackson, personal communication). The modified form of the inhibitor has a reduced affinity for heparin and is unable to inhibit thrombin [3].

The limited proteolysis by thrombin that produces the modified form of antithrombin has been characterized for the bovine enzyme and inhibitor [3,4]. The proteolytic modification was found to be restricted to the scission of a single peptide bond near the $CO_2H$-terminus of the inhibitor, giving rise to two disulfide-linked fragments ($M_r$ ~50 000 and ~5000). Since the amino acid sequence of bovine antithrombin is only fragmentarily known [4,5], the position of the cleavage site was deduced from a homology between the $NH_2$-terminal sequence of the smaller fragment and a segment of human antithrombin starting at Ser-386 [4]. The same cleaved inhibitor as that generated in free form is also released from the antithrombin–thrombin complex by nucleophilic agents [4,6–8] and dissociates spontaneously from the complex [9]. These findings have led to the conclusion that the cleavage site identified in bovine antithrombin is the active site of the inhibitor, i.e., the functional interactive site for protease inhibition [4,7,9].

The nearly complete amino acid sequence and the structure of the 4 identical oligosaccharide chains of human antithrombin have recently been published [10,11]. Because of this comprehensive structural knowledge, and also in order to investigate the generality of our previous findings, we have now extended our studies to include the reaction between human antithrombin and human α-thrombin. A single cleavage site was identified between Arg-385 and Ser-386, i.e., at a position homologous to that observed for the bovine proteins [4].

## 2. Materials and methods

Human antithrombin was isolated by affinity chromatography on heparin–agarose [12]. Human α-thrombin was prepared from fraction III paste [13,14] and was crystallized from 44% saturated $(NH_4)_2SO_4$ in the final preparative step (B. H. Landis and J. W. F. ii, unpublished). The preparation used had a specific clotting activity of 3300 US 'NIH' units/mg protein [13,14], was 100% active by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate [13,15] and consisted of 92.5% α-, 0.8% β- and 6.7% γ-thrombins by SDS–polyacrylamide gel electrophoresis of the material labelled with [$^{14}$C]diisopropylphosphorofluoridate [14]. The thrombin-modified form of human antithrombin was prepared by a modification (I. B. and W. W. Fish, unpublished) of the previous method for the bovine protein [3].

Sequential $NH_2$-terminal analyses were performed

in a Beckman 890 C sequencer with the use of polybrene and a 0.1 M Quadrol program, as in [4]. Phenylthiohydantoin amino acid derivatives were identified by high-performance liquid chromatography and thin-layer chromatography [4].

## 3. Results and discussion

The yield of the human modified antithrombin, prepared with human α-thrombin, was ~60% of that of the bovine modified inhibitor, prepared by the same method with the bovine enzyme. The isolated human modified antithrombin behaved identically to the bovine protein in SDS–polyacrylamide gel electrophoresis [3]; i.e., it co-migrated with intact human antithrombin under non-reducing conditions but migrated slightly faster than the intact protein under reducing conditions. The difference in mobility corresponded to an $M_r$ difference of ~5000. Urea–polyacrylamide gel electrophoresis [3] of the reduced and alkylated human protein showed the presence of a small peptide with the same mobility as the peptide derived from modified bovine antithrombin [3]. These results clearly indicate that the limited proteolytic cleavage of human antithrombin by human α-thrombin is analogous to that previously reported for the bovine system.

The thrombin cleavage site in human antithrombin was identified by direct $NH_2$-terminal sequence analysis of the isolated modified protein without reduction or separation of its fragments. About 65 nmol of protein were analysed; the initial coupling was ~70% and the repetitive yield ~94%. In each cycle, two phenylthiohydantoin amino acid derivatives were recovered in essentially equal amounts, except in cycles 4 and 13, where single derivatives were obtained in about double the normal yield (table 1). Additional derivatives were not detected in any cycle, indicative of only a single proteolytic cleavage. Comparison with the nearly complete sequence of human antithrombin [10] established that the residues identified correspond to those of the $NH_2$-terminal sequences of the intact protein and of a fragment of this protein resulting from the cleavage between Arg-385 and Ser-386. Cleavage at this site produces an $NH_2$-terminal fragment comprising ~385 residues and a 39-residue $CO_2H$-terminal fragment, which are joined by a single disulfide bridge (fig.1).

The site at which human α-thrombin cleaves human antithrombin is thus in a position homologous to that shown [4] for the cleavage of the bovine inhibitor by the bovine enzyme. This homology provides additional, strong evidence that the observed cleavage site is the active site of the inhibitor; i.e., the functional interactive site for protease inhibition [4,7,9]. The proposed location of this site is supported by demonstrations of a homologous active site in the $CO_2H$-terminal region of human $\alpha_1$-protease inhibitor [16,17].

In our studies with bovine antithrombin, the liberation of arginine as the $CO_2H$-terminal residue of the larger fragment of the modified inhibitor could only be inferred from sequence homology with human antithrombin [4]. The present identification of the Arg-385/Ser-386 cleavage site within the reported amino acid sequence of the human inhibitor [10] clearly establishes the participation of an arginine at the thrombin-interactive site. This is in agreement with earlier chemical modification studies [2] and with the recent demonstration of the release of an arginine by carboxypeptidase B digestion of antithrombin–thrombin complex which had been dissociated with methoxyamine hydrochloride [8].

Immediately preceeding Arg-385 in human anti-

Table 1
Sequential $NH_2$-terminal analysis of human modified antithrombin, compared to the reported amino acid sequence of the intact human protein [10]

| Cycle | Residues identified | | Known structure of intact human antithrombin [10] at tentative postitions 1–13 and 385–398 | | | |
|---|---|---|---|---|---|---|
| | | | | | 385 | Arg |
| 1 | His(H) | Ser(H,T) | 1 | His | | Ser |
| 2 | Gly(H,T) | Leu(H,T;38) | | Gly | | Leu |
| 3 | Ser(H,T) | Asn(H,T) | | Ser | | Asn |
| 4 | Pro(H,T;68) | | | Pro | | Pro |
| 5 | Val(H,T;37) | Asn(H,T) | 5 | Val | 390 | Asn |
| 6 | Asp(H,T) | Arg(H) | | Asp | | Arg |
| 7 | Ile(H,T;34) | Val(H,T;39) | | Ile | | Val |
| 8 | (Cys)(T) | Thr(H,T) | | Cys | | Thr |
| 9 | Thr(H,T) | Phe(H,T;27) | | Thr | | Phe |
| 10 | Ala(H,T;29) | Lys(H,T) | 10 | Ala | 395 | Lys |
| 11 | Lys(H,T) | Ala(H,T;28) | | Lys | | Ala |
| 12 | Pro(H) | Asn(H,T) | | Pro | | Asn |
| 13 | (Arg)(H) | | | Arg | | Arg |

Letters in parentheses indicate methods for identification of phenylthiohydantoin derivatives: H, high-performance liquid chromatography; T, thin-layer chromatography. Numbers give nmol derivatives recovered in cycles where quantitation was reliable. About 65 nmol protein were analysed

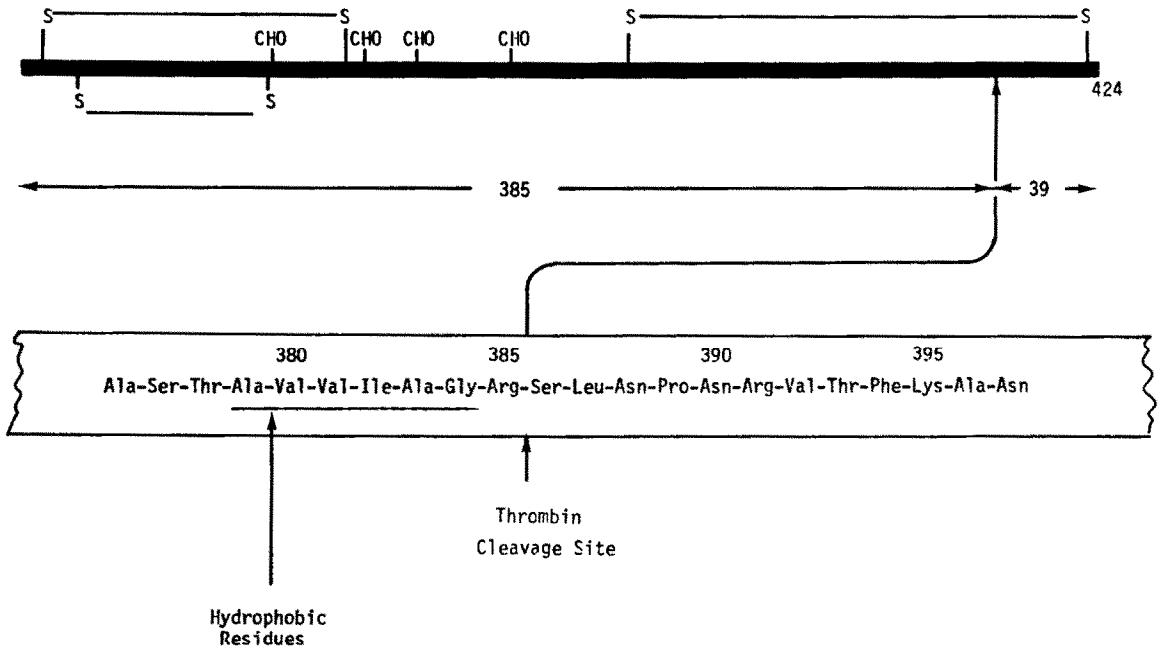


Fig.1. The position of the identified thrombin cleavage site within human antithrombin. The gross polypeptide structure, assigned disulfide bridges (-S–S-) and carbohydrate attachment sites (CHO) are from [10]. The sequence surrounding the thrombin cleavage site is shown, and the hydrophobic residues of the hexapeptide preceeding the cleavage site are underlined.

thrombin is a unique hydrophobic hexapeptide (Ala-379 through Gly-384; fig.1). This hydrophobic peptide may be of functional importance in that it may contribute stability to the antithrombin–thrombin complex by apolar associations with complementary hydrophobic sites in thrombin. A variety of evidence strongly suggests that such sites (e.g., for indole or proflavine binding and for binding of hydrophobic residues preceeding the arginine in synthetic tripeptide substrates) are situated near the catalytic site in thrombin and most likely correspond to the $NH_2$-terminal side of a thrombin-susceptible bond [18,19]. These sites exist both in α-thrombin with high clotting activity and in non-clotting, autoproteolytically derived β- and γ-thrombins [20–22]. This is in contrast to certain active-site regions for fibrinogen recognition, which are found only in procoagulant α-thrombin [20]. The conservation of such hydrophobic sites in the 3 thrombin forms may explain why antithrombin reacts similarly with all these forms [20,21]. Moreover, the accelerating effect of heparin on the antithrombin–thrombin reaction may involve a conformational change of antithrombin [2,23,24], by which the hydrophobic peptide and the contiguous Arg-385/Ser-386 bond become more accessible for interaction with thrombin. In support of this hypothesis, factor $X_a$ does not share with thrombin the same affinity for hydrophobic residues preceeding arginine in synthetic substrates [19,22] and does not react as rapidly as thrombin with antithrombin in the presence of heparin [25].

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