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Site-directed mutagenesis of alanine-382 of human antithrombin III

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Antithrombin III Hamilton is a structural variant of antithrombin III (AT-III) with normal heparin affinity but impaired serine protease inhibitory activity. The molecular defect of AT-III-Hamilton is a substitution of threonine for alanine at amino acid residue 382. Recently it has been shown that both plasma-derived and cell-free-derived AT-III-Hamilton polypeptides act as substrates rather than inhibitors of thrombin and factor Xa. In the present study, the cell-free expression phagemid vector pGEM-3Zf(+)-AT-III$_{1-432}$ was mutated at amino acid residue 382 of AT-III to generate 7 cell-free-derived variants. All these cell-free-derived AT-III variants were able to bind heparin as effectively as cell-free-derived normal AT-III. In terms of α-thrombin inhibitory activity each variant reacted differently. Variants could be grouped into 3 categories with respect to thrombin-AT-III complex formation: (1) near normal activity (glycine, isoleucine, leucine, valine); (2) low activity (threonine, glutamine); (3) no detectable activity (lysine). These data suggest that mutations at position 382 of AT-III may have a variable effect on protease inhibitory activity, depending on either the stability of the P_{12}–P_9 region of the exposed loop of AT-III, or the inability of the amino acid residue at position 382 to interact with a conserved hydrophobic pocket consisting of phenylalanine (at positions 77, 221 and 422) and isoleucine (position 412) residues.

Antithrombin III; Heparin; α-Thrombin

1. INTRODUCTION

Human antithrombin III (AT-III) is a single-chain plasma glycoprotein consisting of 432 amino acids. It is the major physiological inhibitor of thrombin as well as other serine proteases of the intrinsic coagulation pathway [1,2]. AT-III shares both structural and functional homology with other members of the superfamily of proteins known as the serine protease inhibitors, or serpins.

The physiological importance of AT-III is demonstrated by individuals with hereditary AT-III deficiency who often suffer from recurrent thromboembolic disease [3,4]. Characterization of the genetic defects in patients with AT-III deficiency indicates that certain deficiencies can be attributed to point mutations within the AT-III gene. One such variant, AT-III-Hamilton, contains threonine substituted for alanine at amino acid residue 382 [5]. Recently AT-III-Hamilton has been demonstrated to act as a serine protease substrate, but not as an inhibitor [6]. In this communication, we report on site-directed mutagenesis of amino acid residue 382 and its effect on the functional activity of AT-III.

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2. MATERIALS AND METHODS

2.1. *Materials*

All restriction enzymes, T4 DNA polymerase, T4 DNA ligase and T7 RNA polymerase were purchased from either Pharmacia LKB Biotechnology (Baie d'Urfé Quebec) or Bethesda Research Laboratories (Burlington, Ontario). Modified T7 DNA polymerase was purchased from U.S. Biochemicals (Cleveland, OH). The cell-free expression vector pGEM-3Zf(+) and rabbit reticulocyte lysate (RRL) were purchased from Promega-Biotec (Toronto, Ontario). Human thrombin (>3000 NIH units/mg; >99% active) was kindly provided by Dr J. Fenton (New York State Division of Biologicals, Albany, NY). All other chemicals and reagents were of the highest quality available.

2.2. *Site-directed mutagenesis of Ala-382*

E.coli DH5αF′ cells containing the pGEM-3Zf(+)-AT-III$_{1-432}$ construct [7] were infected with M13K07 helper phage to yield single-stranded DNA for site-directed mutagenesis. Site-directed mutagenesis was performed essentially as described by Taylor et al. [8] using the Amersham (Oakville, Ontario) mutagenesis kit. The oligodeoxyribonucleotide primers were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, Ontario). The mixed 20-mer oligodeoxyribonucleotide primer 5′-GGCAGTGAANNAGCTGCAAG-3′ complimentary to the Ala-382 region was synthesized such that the first two nucleotide positions (represented by the letter N), corresponding to the Ala-382 codon, could be altered randomly. The second position, however, corresponding to the Ala-382 codon contained either nucleotides A, G, or T. The AT-III-Hamilton variant was synthesized as previously described [6]. After duplex formation and phosphorothioate enrichment, the plasmid DNA was transformed into *E. coli* DH5α cells. Authenticity of these mutations was confirmed by double-stranded DNA sequencing using the chemically modified T7 DNA polymerase method of Tabor and Richardson [9]. The 18-mer oligodeoxyribonucleotide primer 5′-GTCTCAGATGCATTCCAT-3′ was used for sequencing and is situated 36 bp from the first nucleotide position encoding Ala-382 of AT-III.

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2.3. *Cell-free expression of normal and variant forms of AT-III*

Cell-free transcription-translation was carried out as previously described [7]. The translation mixtures containing the cell-free derived AT-III polypeptides labelled with [^{35}S]methionine were diluted two-fold in 0.04 M Tris-HCl, pH 7.5, 0.3 M NaCl, centrifuged at 150000 × g (Beckman TL-100 ultracentrifuge) for 1 h at 4°C against 0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl. The translation mixtures were then used immediately.

2.4. *Heparin affinity and thrombin complex formation of cell-free-derived normal and variant forms of AT-III*

The ability of cell-free-derived AT-III polypeptides to bind heparin and form covalent complexes with α-thrombin were assessed as described previously [7]. The [^{35}S]methionine-labelled AT-III polypeptides were analyzed by SDS-PAGE [10] under reducing conditions [11] followed by fluorography. Densitometric scanning of the autoradiograms was performed using the Hoeffer GS300 transmittance/reflectance scanning densitometer and the GS350 data system (Hoeffer Scientific, San Francisco, CA).

3. RESULTS

3.1. *Identification of the AT-III variants at amino acid residue 382*

Following site-directed mutagenesis, double-stranded plasma DNA isolated from 24 randomly selected transformed colonies containing the 1.5-kb AT-III cDNA insert was sequenced. Site-directed mutagenesis resulted in the synthesis of 6 unique amino acid substitutions at position 382. The 6 unique substitutions were glutamine, glycine, isoleucine, leucine, lysine and valine. The AT-III-Hamilton variant (alanine-382 → threonine) has been synthesized previously [6]. An amino acid substitution of aspartic acid, which represented one of the possible substitutions, was not identified. In addition, there were no stop codon substitutions identified at position 382. All of the cell-free-derived 382-variants of AT-III were expressed efficiently and had the same apparent molecular mass as cell-free-derived normal AT-III (47 kDa), described previously [7].

3.2. *Heparin-Sepharose chromatography*

The cell-free-derived normal AT-III$_{1-432}$ and 382-variants of AT-III showed similar heparin binding profiles. Densitometric scanning revealed that approximately 60–70% of the cell-free-derived normal, and the 382-variants of AT-III bound to the heparin-Sepharose column and eluted as a major peak between 0.2–0.7 M NaCl (data not shown). This is also consistent with previously published data [7].

3.3. *Thrombin-AT-III (TAT) complex formation*

To compare the protease inhibitory activity of the 7 cell-free-derived 382-variants of AT-III, a 100-molar excess of human α-thrombin was added to the translation mixtures (containing the radiolabelled AT-III polypeptides). Under the conditions used, thrombin-AT-III (TAT) formation peaked at approximately 10% within 2 min. Beyond this time point, however, the complexes dissociated (data not shown). Table I summarizes the relative amount of TAT complexes formed after 2 min by the cell-free-derived 382 variants and α-

Table I

Properties of the AT-III 382-variants

Cell-free derived 382-variants of AT-III were incubated with a 100-molar excess of human α-thrombin at 37°C. Aliquots of the reaction mixtures were terminated with PPACK and the radiolabelled protein bands analyzed by SDS-PAGE followed by fluorography. TAT formation at 2 min. incubation was quantitated by densitometric scanning of the autoradiogram and normalized to wild type cell-free-derived TAT complexes. Hydrophobicity of a particular amino acid residue was determined by its relation between area buried upon folding and standard state area [14].

Amino acid substitution	Side group structure	Residue[a] volume(Å^3)	Hydrophobicity[b]	Relative (TAT)[c] Formation
Ala (wt)[d]	$-CH_3$	92	-	1
Ile	$-CH(CH_3)-CH_2-CH_3$	169	--	0.83
Leu	$-CH_2-CH(CH_3)-CH_3$	168	--	0.52
Val	$-CH(CH_3)-CH_3$	142	--	0.73
Gly	$-H$	66	+,0,-	0.61
Gln	$-CH_2-CH_2-C(=O)-NH_2$	161	+	0.21
Thr	$-CH(OH)-CH_3$	122	0	0.09
Lys	$-CH_2-CH_2-CH_2-CH_2-NH_3^+$	171	+ +	<0.008

(--) strongly hydrophobic; (-) moderately hydrophobic; (0) moderately polar; (+) hydrophilic; (+ +) strongly hydrophilic
[a]Ref. 12,13 [b]Ref. 14 [c]TAT, thrombin-AT-III complex [d]wt, wild type

thrombin; normalized to cell-free-derived normal AT-III. The data indicate that normal cell-free-derived AT-III formed the most TAT complexes within 2 min. The substitution of alanine-382 by the strongly hydrophobic residues isoleucine, leucine or valine produced 0.83, 0.52 and 0.73 times the amount TAT formed by the cell-free-derived normal AT-III, respectively. The neutral substitution glycine, produced 0.61 times the amount of TAT produced by cell free-derived normal AT-III while the slightly hydrophilic substitution glutamine resulted in 0.21 times the amount. The substitution of threonine produced only 0.09 times the amount of TAT produced by cell-free-derived normal AT-III. However, the substitution of the strongly hydrophilic residue lysine resulted in no detectable TAT complex formation after 2 min. The addition of unfractionated heparin at 0.2 U/ml increased the rate of TAT formation but did not increase the relative amount of TAT formed.

The data presented in Table I indicate that amino acid residue volume effects are minimal. The substitutions of isoleucine, leucine or valine at position 382, which are approximately 35–40% larger than alanine, gave similar amounts of TAT complexes to that obtained with cell-free-derived normal AT-III. However, the substitution of lysine at position 382, which is highly hydrophilic and has approximately the same residue volume as isoleucine and leucine, caused a dramatic decrease in TAT complex formation. Despite its impaired thrombin inhibitory activity this cell-free-derived variant was readily cleaved by α-thrombin (presumably at the reactive centre of AT-III), as were all the other variants.

4. DISCUSSION

Recently, the natural variant AT-III-Hamilton (alanine-382 → threonine) was shown to act as a substrate rather than an inhibitor of α-thrombin and factor Xa [6]. This was observed also with cell-free-derived AT-III-Hamilton [6]. This data, together with the results previously reported for AT-III-Charleville [15], AT-III-Cambridge [16] and AT-III-Sudbury [17], which contain the substitution of alanine to proline at position 384, strongly suggest that substitutions at or near position 382 alter the reactive centre loop such that AT-III functions as a substrate, but not as an inhibitor of the serine proteases. The mechanism(s) responsible for this inhibitor to substrate conversion have not been precisely determined. We report herein on the site-directed mutagenesis of codon 382 of AT-III and examine the effect of the substitution of various amino acid residues on the functional activity of cell-free-derived AT-III.

The heparin affinity profiles of cell-free-derived normal AT-III and the 382-variants eluted off heparin-Sepharose were indistinguishable from each other. This is in agreement with previously published data indicating that the naturally occuring variant, AT-III-Hamilton, had heparin binding properties identical to plasma-derived normal AT-III [5]. Thus, amino acid substitutions at position 382 do not appear to alter the ability of AT-III to bind to heparin.

Rose et al. [14] showed that the average area of an amino acid residue buried within a known globular protein, such as AT-III, could be accurately correlated with

	P_{15}	P_{14}	P_{13}	P_{12}	P_{11}	P_{10}	P_9	P_8	P_7	P_6	P_5	P_4	P_3	P_2	Reactive Center ↓ P_1	P'_1	P'_2	P'_3	P'_4	P'_5
1. Antithrombin III	Gly	Ser	Glu	Ala	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Ala	Gly	Arg	Ser	Leu	Asn	Pro	Asn
2. Heparin Cofactor II	Gly	Thr	Gln	Ala	Thr	Thr	Val	Thr	Thr	Val	Gly	Phe	Met	Pro	Leu	Ser	Thr	Gln	Val	Arg
3. Alpha-1-Antiplasmin	Gly	Val	Glu	Ala	Ala	Ala	Ala	Thr	Ser	Ile	Ala	Met	Ser	-	Arg	Met	Ser	Leu	Ser	Ser
4. Plasminogen Activator Inhibitor	Gly	Thr	Val	Ala	Ser	Ser	Ser	Thr	Ala	Val	Ile	Val	Ser	Ala	Arg	Met	Ala	Pro	Glu	Glu
5. Protein C Inhibitor	Gly	Thr	Thr	Ala	Ser	Ser	Asp	Thr	Ala	Ile	Thr	Leu	Ile	Pro	Arg	Asn	Ala	Leu	Thr	Ala
6. Alpha-1 Antitrypsin	Gly	Thr	Glu	Ala	Ala	Gly	Ala	Met	Phe	Leu	Glu	Ala	Ile	Pro	Met	Ser	Ile	Pro	Pro	Glu
7. Chymotrypsin Inhibitor	Gly	Thr	Glu	Ala	Ser	Ala	Ala	Thr	Ala	Val	Lys	Ile	Thr	Leu	Leu	Ser	Ala	Leu	Val	Glu
8. C1-Inhibitor	Gly	Val	Glu	Ala	Ala	Ala	Ala	Ser	Ala	Ile	Ser	Val	Ala	Arg	Thr	Leu	Leu	Val	Phe	Glu
9. Ovalbumin	Gly	Arg	Glu	Val	Val	Gly	Ser	Ala	Glu	Ala	Gly	Val	Asp	Ala	Ala	Ser	Val	Ser	-	Glu
10. Angiotensinogen	Glu	Arg	Glu	Pro	Thr	Glu	Ser	Thr	Gln	Gln	Leu	Asn	Lys	Pro	-	-	-	-		-

Fig. 1. Sequence alignment of the reactive centre loops of ten members of the serpin family. The first 8 members, including AT-III, are inhibitory serpins. Ovalbumin and angiotensinogen are non-inhibitory members of the serpin family. The boxed region shows the extent of conservation of small side-chain amino acids at P_9–P_{12} amongst the active serpins. Note that this conservation is not maintained in the non-inhibitory serpins ovalbumin and angiotensinogen. The amino acid sequences of the serpins were aligned for maximum homology.

hydrophobicity. The results presented here suggest that changes in hydrophobicity at position 382 of AT-III play a role in the formation of stable inhibitory complexes with α-thrombin. Replacement of alanine-382 by strongly hydrophobic residues resulted in comparable amounts of TAT complex formation to that obtained with cell-free-derived normal AT-III. Size optimum effects appear to be minimal since isoleucine, leucine and valine, which are approximately 35–45% larger than alanine, gave TAT formation values similar to wild type cell-free-derived normal AT-III. However, the replacement of alanine-382 by the strongly polar lysine residue completely eliminated TAT formation. Although lysine and the strongly hydrophobic residues (isoleucine, leucine, valine) have similar residue volumes, the hydrophobic differences between them are considerable. This change in hydrophobicity may, in part, explain the dramatic drop in TAT formation amongst these variants and is supported by previously reported data which are discussed below.

Amongst the inhibitory serpins, the region P_{12}–P_9 (in AT-III this is represented by residues 382–385) consists of small side-chain amino acids, principally alanine, glycine and serine (Fig. 1). It has recently been suggested that this highly conserved region is necessary to provide flexibility of the N-terminal stalk of AT-III such that it has the ability to act as an inhibitory serpin [18,19]. It is postulated that once the reactive centre of AT-III has been cleaved by the serine protease, this N-terminal stalk of AT-III moves towards the underlying β-sheet (due to the flexible nature of the 382–385 region) thereby allowing complex formation to occur. This concept is supported by the observations that the non-inhibitory serpins ovalbumin and angiotensinogen, considered to have large bulky side-chain residues in this region, fail to undergo the S-R transition, typical of the inhibitory serpins [20]. Thus, the substitution of threonine for alanine at position P_{12} in AT-III [5], which has been shown to convert AT-III into a substrate rather than an inhibitor of serine proteases [6], may act to after the flexibility of the P_{12}–P_9 region by interacting with additional adjacent amino acids via hydrogen bonding. The crystal structure of the cleaved form of the inhibitory serpin α_1-antitrypsin (α_1-AT) shows that the alanine at position P_{12}, which is analogous to alanine-382 in AT-III, fits tightly into a hydrophobic pocket consisting of phenylalanine residues at position 51,190 and 384, and a methionine residue at position 374 [19,20]. When the amino acid sequences of AT-III were aligned for maximum homology with α_1-AT, phenylalanine residues at positions 77, 221 and 422 in AT-III lined up exactly as in α_1-AT. The methionine residue at position 374 in α_1-AT is replaced by an isoleucine residue at the corresponding position 412 in AT-III. In addition, lysine and arginine residues located at positions 222 and 413 respectively, in AT-III would be positioned in close proximity to this hydrophobic pocket (the lysine residue at position 191 of α_1-AT is also conserved in AT-III). Thus, strongly hydrophobic amino acid substitutions at position 382 in AT-III would result in the maintenance of serine protease inhibitory activity. The substitution of lysine at position 382 in AT-III likely would prevent movement of the cleaved N-terminal stalk into the β-sheet, as described previously [18,19]. However, a more probable explanation is that the strongly hydrophilic nature of lysine, combined with its positive charge (at physiological pH) would not allow the N-terminal stalk to interact with the hydrophobic pocket in AT-III as described above [18–20]. Consequently, the substitution of lysine at position 382 would convert AT-III from an inhibitor of thrombin to a substrate. Furthermore, it is interesting to note that both the non-inhibitory serpins, ovalbumin and angiotensinogen, contain an arginine residue at position P_{14} (Fig. 1). This, however, is not the case for the inhibitory serpins. This may imply that the presence of an arginine residue at this position could contribute to the non-inhibitory nature of serpins.

The data presented here assumes that AT-III undergoes a similar S-R transition as described for α_1-AT [20]. Confirmation of the positioning of the alanine 382 residue in the P_{12}–P_9 region of AT-III, before and after cleavage by the target protease, must, however, await crystallographic data. Nonetheless, the present data suggest that impairment of serine protease inhibitory activity by mutations at position 382 in AT-III result from either: the increased stability (rigidity) of the P_{12}–P_9 region of the exposed reactive centre loop or; the inability of the amino acid residue at position 382, upon cleavage, to interact with the conserved hydrophobic pocket consisting of three phenylalanine residues (at positions 77, 221 and 422) and an isoleucine (position 412) residue.

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