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Site-directed mutagenesis of the P2 residue of human antithrombin

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Abstract

Antithrombin (AT) is the principal inhibitor of thrombin in human plasma, and a member of the serine proteinase (serpin) family of proteins. Previously, we have described a point mutation in the human AT gene that converted amino acid 392 from glycine to aspartic acid which was associated with thrombotic disease in a Swedish family [(1992) Blood 79, 1428–1434]. This observation prompted us to investigate the consequences of other substitutions at this position, termed P2 with respect to the reactive centre. Site-directed mutagenesis was employed to generate seven mutants (Pro, Met, Gln, Val, Lys, Glu, and Asp), whose properties were compared with wild-type recombinant AT, following in vitro transcription and cell-free expression in a rabbit reticulocyte lysate system. With only one exception, the variant forms were less active than the wild-type in forming complexes with either α-thrombin, factor Xa, or trypsin. Hydrophobic (Val) or negatively charged (Asp or Glu) substitutions were particularly disruptive, in that these variants exhibited less than 10% wild-type antithrombin or antitrypsin activity. In contrast, the formation of complexes with the various proteases of the Pro variant was essentially unimpaired. We conclude that the P2 residue of AT plays a role in optimal presentation of the reactive centre to its cognate protease, and propose that the observed requirement of Gly or Pro at this position is suggestive of a bend in the polypeptide backbone that aids in this presentation.

Key words: Antithrombin; Thrombin; Serpin

1. Introduction

Antithrombin (AT), or antithrombin III, is the principal natural inhibitor of thrombin and other procoagulant serine proteases found in plasma [1,2]. This 60 kDa glycoprotein is a suicide inhibitor of its cognate proteases, and a member of the serine proteinase (serpin) family of proteins [3,4]. Attack by the protease at the reactive centre of AT, Arg^{393}–Ser^{394}, leads not only to cleavage of AT, but also to the formation of a stable AT-protease complex [5]. In the complex, a covalent ester bond links the carboxyl group of AT residue Arg^{393} and the hydroxyl group of the active site serine of the protease [6].

Congenital deficiencies of AT are associated with an increased risk of thrombotic disease [7,8]. Qualitative (type II) deficiencies result from alterations to single amino acid residues in the protein product of the mutant allele [9]. Recently, we identified a novel mutation of AT, G392D, in a Swedish kindred with familial thrombosis [10], AT-Stockholm. In the present study, we examined the consequences of substitutions other than Asp at this position, a single residue N-terminal to the reactive centre, by site-directed mutagenesis and expression in a cell-free system. Our results suggest that Gly or Pro residues are required at this location for optimal inhibition of both thrombin and two other serine proteases, perhaps because of the helix-breaking potential of these amino acids.

2. Materials and methods

2.1. Materials

Human α-thrombin (> 2500 NIH units/mg; > 99% active) was kindly provided by Dr. J. Fenton (New York State Division of Biologicals, Albany, NY). Human Factor Xa was the generous gift from Dr. F.A. Ofosu, McMaster University, while trypsin was purchased from Sigma Chemical Company (St. Louis, MO). All restriction, DNA modifying enzymes, and T7 sequencing kits were from Pharmacia LKB Biotechnology (Baie d'Urfé, QC). Bethesda Research Laboratories (BRL) (Burlington, ON) was the supplier of *E. coli* DH5α cells competent for transformation. Promega Biotec (Madison, WI), was the source of rabbit reticulocyte lysate. A kit for site-directed mutagenesis was purchased from Amersham (Oakville, ON). The thrombin inhibitor D-phenylalanyl-L-prolyl-arginine chloromethyl ketone (PPACK) (> 99% active) was purchased from Calbiochem (Mississauga, ON), and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) from Sigma (St. Louis, MO). Translation grade [^{35}S]methionine (>1000 Ci/mmol, 10 μCi/μl) was purchased from New England Nuclear (Lachine, QC). Protein molecular weight standards were from Bio-Rad Laboratories (Oakville, ON). Oligonucleotides were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, ON. All other chemicals and reagents were of the highest quality available.

2.2. Site-directed mutagenesis

The previously described [11] phagemid pGEM-3Zf(+)-AT-III_{1-432} was used to provide single stranded starting material for mutagenesis, which was carried out using the gapped duplex method in accordance with the kit manufacturer's instructions (Amersham). The degenerate

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oligodeoxynucleotide primers 5′-TGATTGCT(ACG)(ACT)(ACGT)-CGTTCGCT-3′ and 5′-TGATTGCT(AC)(ACT)(AG)CGTTCGCT-3′ were employed, and individual clones were characterized by double-stranded sequencing of the target codons and neighbouring DNA. Resulting plasmids differing from wild-type pGEM3zf(+)ATIII$_{1-432}$ at position 392 were designated pGEM3zf(+)ATIII$_{1-432G392X}$, where X represents the single-letter code for the new amino acid residue.

2.3 In vitro transcription and cell-free translation

In vitro transcription reactions employing T_7 polymerase were performed as described [12,13], following linearization of plasmid templates with *Eco*RI. The resulting AT-derived mRNA transcripts were translated in an mRNA-dependent rabbit reticulocyte lysate as described previously [12,13], except that the reaction mixture was supplemented with oxidized glutathione to a final concentration of 2.5 mmol/l.

2.4 Protease-complexing assays

The reaction of thrombin with cell-free derived AT polypeptides was monitored as described previously [12,13]. Briefly, in vitro translation mixtures were diluted 1:1 with TBS, dialyzed overnight against the same buffer, aliquoted and flash-frozen at −70°C. Following determination of the trichloroacetic acid precipitable counts, concentrations of translated AT protein species were normalized by the addition of translation mixtures to which no RNA had been added, and reacted with thrombin or factor Xa at a final concentration of 1.0 μM. Trypsin was employed at a final concentration of 200 nM, in order to maximize complex formation and minimize cleavage at sites other than the reactive centre. Reactions were stopped by the addition of PPACK in the case of thrombin, and PMSF in the case of factor Xa and trypsin.

2.5 Gel analysis and densitometry

Electrophoretic separation of radiolabeled proteins under reduced or non-reduced conditions, and fluorography of dried gels was done as previously described [11]. Densitometric scanning of autoradiograms was performed using an Ultroscan XL apparatus (LKB, Bromma, Sweden).

3. Results

3.1. Mutagenesis of AT at residue 392

Site-directed mutagenesis was employed in order to generate forms of the antithrombin cDNA in which codon 392 (GGA, Gly) had been altered. Sequencing of a total of 60 clones, derived from mutagenesis reactions that used two separate mixtures of oligodeoxynucleotides that were degenerate at codon 392, yielded seven different mutants: Asn (AAC), Asp (GAC), Glu (GAA), Lys (AAA), Met (ATG), Pro (CCA), and Val (GTA). The frequency of appearance of the different mutants was as expected, in that those resulting from a single nucleotide mismatch were more common than those resulting from double or triple mismatches.

3.2. Reaction of cell-free derived AT with serine proteases

In order to ascertain the effect of various amino acid substitutions on the biological activity of the AT protein, we exploited a cell-free expression system that has been used extensively in our laboratory for the study of normal recombinant and mutant AT molecules [11–13]. Following in vitro transcription of pGEM-AT-III$_{1-432}$, translation of the resulting mRNA in a messenger-dependent rabbit reticulocyte lysate, in the presence of [^{35}S]methionine, leads to the synthesis of labelled AT polypeptides. These molecules are the major radiolabelled proteins in the reaction mixture which can be visualized following SDS-PAGE and fluorography without the need for fractionation or immunoprecipitation. Addition of excess unlabelled thrombin results in the formation of a covalent thrombin-AT complex, as shown by the appearance of a thrombin-dependent 80 kDA band (Fig. 1, panel A). Similar complexes can be observed following the reaction of cell-free derived AT with both factor Xa (Fig. 1, panel B) and trypsin (Fig. 1, panel C). In the latter case, five-fold less trypsin than the other two serine proteases was employed, in order to minimize cleavage at arginyl residues other than the reactive centre. In addition to the TAT species, in some reactions two other products with more rapid electrophoretic mobility than full-length AT can be seen: a protease independent band that derives from internal inititiation at Met17 which does not contribute significantly to TAT formation, and a protease-dependent band resulting from AT cleavage.

3.3. Reaction of the AT 392 variants with α-thrombin

We first analyzed the reaction with thrombin of the family of AT mutants differing at residue 392. In order

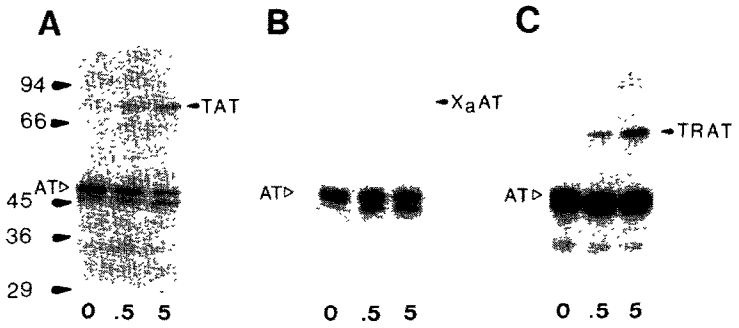

Fig 1. Reaction of cell-free derived AT with serine proteases. ^{35}S-labelled AT molecules were generated in rabbit reticulocyte translation reactions, and reacted for various times (shown below each panel, in minutes) with α-thrombin (Panel A), factor Xa (Panel B), or trypsin (Panel C). Positions and sizes (in kDa) of molecular weight standards are shown at left; positions of thrombin-antithrombin (TAT), factor Xa-antithrombin (XaAT), and trypsin-antithrombin (TRAT) complexes are indicated, as is the position of full-length cell-free derived AT (open arrowheads). Autoradiograms of 10% SDS-polyacrylamide gels are shown.

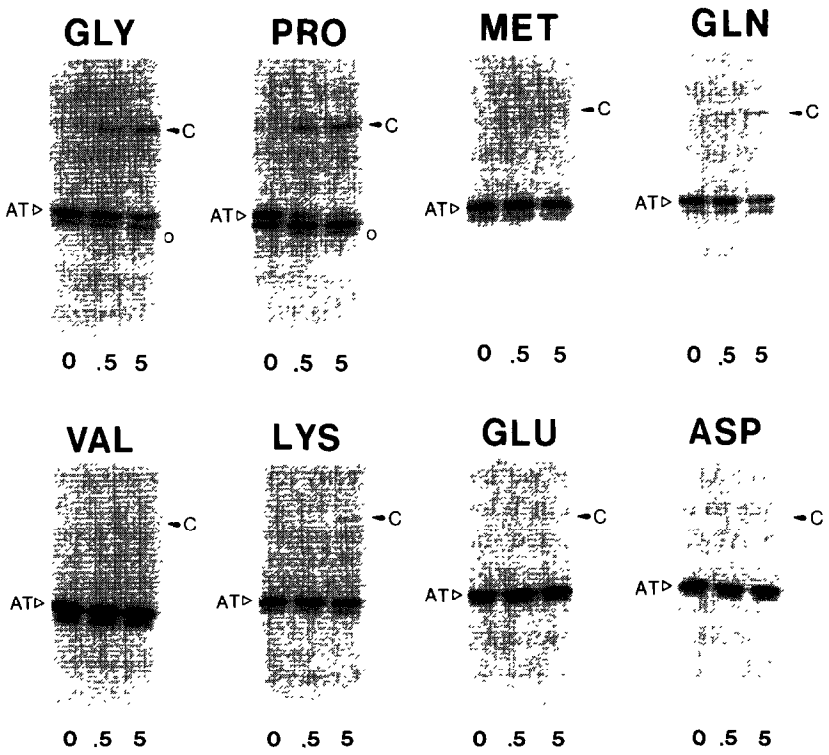

Fig. 2. Reaction of cell-free derived AT variants differing only at residue 392. Cell-free derived AT species containing the various substitutions at residue 392, identified at the top of each panel, were reacted with α-thrombin as in Fig. 1. The migration position of the TAT complex is highlighted (C), as is the position of full-length cell-free derived AT (open arrowheads), and that of thrombin-cleaved AT (open circles).

to minimize variation between batches of reticulocyte lysate and differences between synthesis reactions performed at different times, all eight forms of recombinant human AT were synthesized, diluted, dialyzed, aliquoted and flash-frozen at the same time. Total TCA-precipitable counts were normalized to the lowest incorporation reaction, and AT proteins reacted with excess (1.0 μM) thrombin for 0, 0.5, or 5 minutes. The resulting autoradiograms are shown in Fig. 2, and their quantitation in Table 1.

Comparison of the proportion of the input AT found in TAT complexes at five minutes revealed that the AT mutants could be grouped into three categories, according to their reactivity with thrombin: minimally impaired (> 60% wild-type reactivity); moderately impaired (> 25%, < 60% wild-type reactivity); and severely impaired (< 10% wild-type reactivity). Based on these criteria, the Pro and Gln mutants showed minimal impairment, the Lys and Met variants moderate impairment, and the Asp, Glu, and Val variants severe impairment. Reaction with thrombin can also lead to the production of a cleaved form of AT, with increased mobility under reducing conditions (Fig. 2, open circle). This product was detectable only in reactions with the wild-type recombinant AT or the Pro variant. In the latter case, this product was generated more rapidly, and in greater amounts, than in the former.

3.4. *Reaction of the AT 392 variants with the other serine proteases*

In order to determine if the decreases observed in reactivity with thrombin were specific to the reaction of AT with this procoagulant protease, similar experiments were performed with Factor Xa and trypsin, and the

Table 1
Quantification of AT-protease complex formation

Alteration	% Relative complex formation			Side-chain characteristic	
	IIa	Xa	Trypsin	Average volume	Hydropathy index
None (WT)	100	100	100	66	−0.4
G392 P	81	103	93	129	−1.6
G392 Q	66	62	81	161	−3.5
G392 K	28	43	32	171	−3.9
G392 M	26	21	11	170	1.9
G392 D	10	4	10	125	−3.5
G392 V	7	10	11	142	4.2
G392 E	7	35	7	155	−3.5

Experiments identical to those depicted in Fig. 2 were performed, except that either thrombin (IIa), factor Xa, or trypsin were employed. Complex formation after 5 min of incubation was determined by densitometric scanning of autoradiograms, and the results expressed as % complex formation relative to that of the cell-free derived wild-type AT. Side-chain volume (in cubic angstroms) and hydropathy index (arbitrary units) are from [19] and [20] respectively.

results quantified (Table 1). Very similar results to those seen with thrombin were observed in the case of trypsin, in terms of the ranking of variants and the extent of mutant inhibitor dysfunction. The results with Factor Xa were also similar, with the exception that the Glu substitution affected this reaction to a lesser extent than for the other two proteinases. The reactivity of the Pro variant with either factor Xa or trypsin was essentially unchanged relative to wild type.

4. Discussion

We have investigated the effects of amino acid substitutions at the P2 position of AT. Previously we had shown that altering this residue to Asp resulted in a loss of AT function, both in an AT-deficient kindred and with the equivalent cell-free-derived recombinant product [10]. The present study has demonstrated that not only negatively charged (Asp or Glu) but also hydrophobic (Val), neutral (Met or Gln), or positively charged (Lys) substitutions, at this position, also reduce the ability of AT to form complexes with both thrombin and two other serine protease inhibitors, factor Xa and trypsin.

Examination of Table 1 suggests that the reactivity of the different mutants with their cognate proteases does not correlate with residue size or hydrophobicity. Rather, both charged and strongly hydrophobic residues are not favoured. The wild-type Gly and Pro residues gave the most functional recombinant AT products. Indeed, the Pro variant exhibited more rapid and more extensive cleavage by thrombin than did the wild-type. Densitometric scanning of the full-length AT species present before and after incubation with thrombin showed that only 17% of the Pro variant remained unreacted, as compared to 57% of the wild-type (Fig. 2). However, since protease inhibition depends upon serpin complex formation, our data suggests that the Pro variant is minimally impaired in its function, rather than supranormal, as its cleavage behaviour might suggest.

Interestingly, both Gly and Pro residues are frequently found at β-turns in proteins, and are therefore thought of as helix-breaking residues [14,15]. While neither AT nor any other inhibitory serpin has been crystallized, the crystal structure of the related serpin, ovalbumin, has been solved. The reactive centre loop of the protein is found as a three-turn α-helix, terminating with the P1–P1′ bond [16]. It has been suggested that the inhibitory serpins, although exhibiting mobile conformations of this region, have retained the ability to fold this region into a similar α-helix [17]. If this is the case, then the requirement for helix-breaking residues at P2 becomes obvious. On the other hand, the observed preference for Gly or Pro residues at this position of AT may simply indicate that these residues interact with the active site of the attacking protease most effectively. In this regard, it is known that Phe-Pro-Arg-CH_2Cl is a more efficient inhibitor of thrombin than chloromethyl ketones lacking the Pro residue at P2 [18].

In conclusion, the results presented here suggest that residue 392 of AT (P2) contributes to the optimal presentation of the adjacent reactive centre to its cognate proteases; this presentation may involve the maintenance of a β-turn or other helix-breaking structure. Other substitutions could both obscure this necessary structure and directly repel the incoming protease's active site.

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