

ALTERATION OF THE POSITIVELY CHARGED D-HELIX IN HUMAN ANTITHROMBIN

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ABSTRACT

The serpins antithrombin (AT) and protein C inhibitor (PCI) inhibit thrombin in a reaction that is accelerated by heparin (1000-fold and 40-fold respectively). The heparin binding domains of PCI and AT differ. In AT, the D-helix is a major part of the heparin-binding domain, while the H-helix is the heparin-binding domain in PCI. Compared to antithrombin, PCI is a more potent inhibitor of thrombin bound to thrombomodulin (TM). To explore the role of the antithrombin D-helix in this reaction we changed two positively charged residues in the D-helix (K125 and R129) into neutral and negatively charged amino acids by site-directed mutagenesis. These constructs will then be expressed in baculovirus and assayed for their ability to inhibit thrombin in the presence and absence of heparin and TM.

INTRODUCTION

Antithrombin (AT) and protein C inhibitor (PCI) are members of the serpin superfamily and function as physiological inhibitors of serine proteases, including thrombin, factor XIa, and protein C. These serpins form a bond between their reactive site loop and the active site of their target proteases. Incomplete cleavage of this bond induces a conformational change that traps the serpin, preventing its dissociation and inhibiting its further use (1,5,10). Heparin is a long carbohydrate which binds adjacently to the surface of both AT and thrombin, and can increase the inactivation reaction 1,000- 2,000 fold under optimum conditions (2, 9, 6, 3). Thrombin inhibition by PCI is not as dramatically affected by heparin (only 40-fold) (7). The heparin-binding site of AT is localized in the D-helix whereas heparin binds to the H-helix of PCI. These helices are mainly comprised of basic residues (4, 8). To explore the role of the antithrombin D-helix in this reaction we changed two positively charged residues in the D-helix (K125 and R129) into neutral and negatively charged amino acids by site-directed mutagenesis. These mutant proteins will then be expressed in insect cells with baculovirus(and assayed for their ability to inhibit thrombin in the presence and absence of heparin.

MATERIALS AND METHODS

Site directed mutagenesis. Synthetic oligonucleotide primers, with the desired mutations, were designed to anneal to pGEM-AT. Primers, pGEM-AT, and *Pfu* DNA polymerase were incubated for 16 cycles at 55°C, 95°C, and 68°C to produce the mutagenesis product. The PCR products were digested with *DpnI* to remove the unmutagenized DNA. The products were then sequenced to insure that the correct DNA sequences were obtained (Figure 1).

Subcloning of AT into pVL1392. The pGEM-AT D-helix region was located between the *PstI* and *SacI* restriction endonuclease sites. PVL1392 containing wild type AT was also digested with the same restriction endonucleases. The digestion mixtures were incubated at 37°C for 45 min. The pGEM D-helix fragment was ligated into pVL1392 using DNA ligase and ATP at 12°C overnight.

Homologous recombination. The pVL1392-AT was used in homologous recombination with Baculogold™ DNA, PharMingen, according to the manufacturer’s directions and incubated at 37° C for 72h with *Spodoptera fugiperda* cells (SF9). The virus was then amplified by infection of fibroblast cells from *Tricoplusia ni* (Hi 5 cells) with viral media from the homologous recombination.

Analysis of recombinant AT. The recombinant protein was purified from media using heparin affinity chromatography. The protein was then visualized by immunoblot using the following antibodies: rabbit anti- AT and goat anti-rabbit conjugated with Alkaline Phosphatase. Color development was achieved by incubation in Nitro Blue Tetrazoleum/Bromo Chloro Indoyl Phosphate. The concentration of protein was determined by enzyme linked immunosorbant assay (ELISA) using the same antibodies above, and Para-Nitrophenyl Phosphate as the substrate. The absorbance was read at 405 nm.

Activity Assay. Normal human plasma antithrombin (hum AT), wild-type recombinant antithrombin (rAT) and each of the mutant proteins, each at 100nM, were incubated with thrombin (2 nM), in the presence and absence of heparin (1µg/ml). After the incubation period, a substrate for thrombin was added to measure the amount of thrombin activity remaining.

RESULTS AND DISCUSSION

DNA sequencing of the mutagenized cDNA indicated that the desired mutations had occurred (figure 1). Immunoblot data confirmed that intact proteins were being produced at the correct molecular weight (~ 58 kDa)(Data not shown). The activity assays showed normal activity in human AT and wild type recombinant AT and some activity in all the mutants (Table 1). Stimulation by heparin is significantly lower in the mutants, compared to rAT (Figure 2).

AT	⁴⁶³	TTT GCC AAA CTG AAX TGC CGACTC TAT ⁴⁸⁹
AT1		TTT GCC GAA CTG AAX TGC CAA CTC TAT
AT2		TTT GCC CAA CTG AAX TGC GAA CTC TAT
AT2		TTT GCC CAA CTG AAX TGC CAA CTC TAT
AT12		TTT GCC GAA CTG AAX TGC GAA CTC TAT

Figure 1. Sequence the cDNA for correct mutations. DNA Sequence of AT D-Helix mutants. AAA= Lysine, CGA=Arginine, GAA=Glutamate, CAA=Glutamine.

Table 1: Rate of Thrombin Inhibition by rAT Mutants in the Presence and Absence of Heparin.

	No Heparin	With Herapin	Fold Stimulation
Hum AT	1.00E+04	2.02E+06	202
rAT	3.61E+03	1.02E+06	282
3D3H	9.53E+02	4.25E+04	45
3D4H	1.23E+03	5.78E+04	47
12D3H	1.98E+03	6.28E+04	32
12D4H	2.23E+03	1.36E+05	61

*Rates are in (M⁻¹s⁻¹)

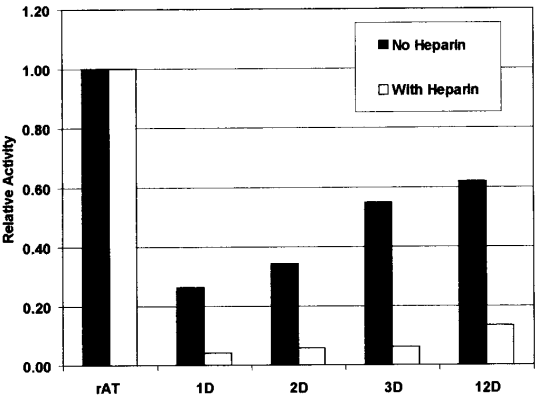


Figure 2. Rate of thrombin inhibition by rAT mutants in relation to wt rAT in the presence and absence of heparin.

The D-helix of AT was successfully changed as shown by the sequenced DNA. AT was produced at the approximate MW of human AT with slight variation due to lack of glycosylation. Analysis of the protein activity shows that while activity is still present in the mutants, they have far less than those of the recombinant and human proteins. When comparing the effect of heparin stimulation on the mutants and recombinant wild type, the mutants show a definite lack of stimulation. This shows that the K125 and R129 sites of the D-helix do affect the ability of heparin to stimulate AT inactivation of thrombin. While heparin stimulation was studied, it is not yet known how these changes affect the binding of heparin to AT. Further research of the mutants will involve study of AT-heparin binding.

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