

Conversion of the Heparin Binding Domain in Human Antithrombin to Resemble that of Protein C inhibitor

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ABSTRACT

The serpins antithrombin (AT) and protein C inhibitor (PCI) both inhibit thrombin in a reaction that is accelerated by heparin. 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. The positively charged D-helix residues K125 and R129 were changed to neutral and negatively charged amino acids by site-directed mutagenesis. In addition the negatively charged H-helix residues D309, E310, E312 and E313 were also changed to neutral and positively charged residues. This generated an AT mutant in which the heparin-binding site has been moved from the D-helix to the H-helix. The mutant proteins will be expressed in baculovirus and purified. These proteins will then be assayed with thrombin in the presence and absence of heparin and thrombomodulin to determine whether the location of the heparin-binding site influences these reactions.

BACKGROUND

A cut or damaged blood vessel would continue to bleed indefinitely if it were not for a process of repair known as hemostasis. Under this process, platelets attach to the surface of the damaged vessel and clump together to form a temporary plug. A series of reactions then occurs to stabilize this plug. Clotting factors convert prothrombin to thrombin, which then converts fibrinogen to fibrin. The fibrin is then cross-linked trapping the blood cells and forming a clot (Stryer, 1996). Thrombin is inhibited by a protein called antithrombin (AT). This plasma-borne protease inhibitor plays a pivotal role in the regulation of hemostasis (Gillispie, 1991). The binding of the carbohydrate heparin to antithrombin is quite specific and involves a unique pentasaccharide sequence that precisely binds to a well-defined site on antithrombin. This induces a conformational change in antithrombin that causes a 1000-fold increase in inhibition of thrombin (Carrell, 1997).

METHODS

All AT mutants were synthesized via a site-directed mutagenesis kit. In the polymerase chain reaction, human AT III was used as a template, while the primers contained the desired mutations. The human AT III template was denatured at 94°C, the primers were annealed to their complementary sequences at 55°C, and the primers were elongated at 72°C. This process was then repeated to achieve amplification.

Plasmids pGEM-AT and pVL1392 were purified from overnight cultures using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The purified pGEM-AT was digested with *Pst* I and *Sac* I restriction endonucleases to remove the D-Helix, while pVL 1392 was digested with *Nco* I and *Bam* HI at 37°C for forty-five minutes. Both helices were ligated into pVL1392 using T4 DNA ligase at 12°C. Colonies were grown and plasmids isolated and the DNA sequenced to identify the correct mutants.

Tricopsulina ni (HI5) cells were grown in EX-Cell Media until confluent. These cells were then used to perform the homologous recombination using a Baculovirus Expression Vector System (PharMingen, San Diego, CA). A CaPO₄ precipitate was introduced, which trapped the baculoviral and pVL1392 AT DNA. The precipitate was then endocytosed by the HI5 cells. The homologous recombination occurred within the nucleus. The recombinant viral protein (rAT) was then secreted out into the media.

The rAT from the media was then purified by Heparin Affinity Chromatography. The rAT was then used to perform an Immunoblot. The SDS-PAGE gel was transferred to nitrocellulose and subjected to a primary and secondary antibody, and then a chromogenic substrate, Bromo-Chloro-Indoyl Phosphate and Nitro Blue Tetrazolium (BCIP/NBT).

Activity assays were performed to study the kinetics of the rAT. Thrombin was added to a microtiter plate with some wells containing either heparin or buffer. The rAT was then added to these wells inhibiting the thrombin. Only thrombin not inhibited by the rAT could cleave substrate. Absorbance was measured at 405nm to determine thrombin activity.

RESULTS AND DISCUSSION

Recombinant antithrombin (rAT) activity was assayed in the presence and absence of heparin. All mutants showed less activity than wild type rAT (wt rAT) and human AT. Human AT (humAT) showed a 202-fold and rAT showed a 282-fold increase in activity in the presence of heparin. However, mutant 3D4H only showed a 15-fold increase and mutant 12D3H showed a 32-fold increase (Table 1). All four rAT mutants possessed activity that was relatively normal in the absence of heparin, however, activity was significantly lower in the presence of heparin when compared to wt rAT and humAT (Figure 1). By changing the charge on the D-helix from positive to either neutral or negative, activity is dramatically lowered in the presence of heparin.

The immunoblot analysis verified that the mutagenized proteins were intact and are being produced by the insect cells, *Tricopsulunia ni*. An immunoblot was also performed which verified that our rAT is being produced at the correct molecular weight (58,000 Da).

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

	No Heparin.	With Herapin	Fold Stimulation
Human AT	1.00E+04	2.02E+06	202
rAT	3.61E+03	1.02E+06	282
3D3H	5.05E+03	1.97E+05	39
3D4H	4.05E+03	6.27E+04	15
12D3H	5.05E+03	1.63E+05	32
12D4H	1.23E+03	2.57E+04	21

Rates are in (M⁻¹s⁻¹)

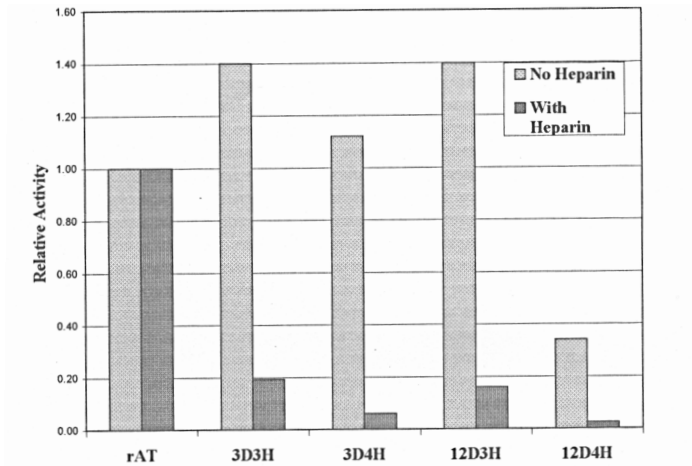


Figure 1 Rate of thrombin inhibition by rAT mutants in relation to wild type rAT in the presence and absence of heparin.

REFERENCES

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