Negatively Charged H-helix in Human Antithrombin

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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. However, 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. Alignment of the sequences of PCI, AT and heparin cofactor II (HCII) suggests that AT is unique in having a negatively charged H-helix, and a methionine at residue 314, which corresponds to residue 278 of PCI. The other serpins have positively charged helices and an arginine at residue 278. In the first mutant, AT-pos (#3), the glutamate and aspartate residues in the H-helix of AT were replaced with lysine, mimicking the structure of the H-helix in PCI. In the second mutant, AT-neut (#4), the glutamate and aspartate residues were changed to uncharged residues, asparagine and glutamine. Both the wildtype and mutant proteins were expressed in baculovirus and purified. These proteins were then assayed with thrombin in the presence and absence of heparin to determine whether these mutations alter the activity of AT.

INTRODUCTION

In the United States, approximately 1.5 million heart attacks occur each year. Thirty percent end in death with more than half of these deaths occurring before the individuals ever reach the hospital (Fauci 1998).

Blood clotting can be beneficial when healing a cut or scrape of the skin. However, blood clotting can be lethal if a clot blocks the flow of blood to the brain (stroke), lungs (embolism), or heart (myocardial infarction). Blood clotting is controlled by a cascade of reactions involving the successive activation of clotting factors. The enzyme thrombin is an essential factor in initiating the final formation of a blood clot. Thrombin activity is closely regulated by AT (AT) and protein C inhibitor (PCI) (Rezaie, et. al., 1995).

Heparin is a carbohydrate that acts as a bridge between thrombin and its inhibitors, AT and PCI. This connection accelerates the reaction between Thrombin and AT or PCI, therefore, stimulating anticoagulation. Heparin contains negatively charged portions that can bind to the positively charged portions on AT and PCI (Rezaie, 1995). The binding site differs on AT and PCI. On AT, the D-helix is the binding site; in contrast, the H-helix is the binding site on PCI. By better understanding how these anticoagulants work, we will be one step closer to creating a drug that could treat and, perhaps, prevent lethal blood clots. This could make heart attacks, strokes, and emboli less frequent.
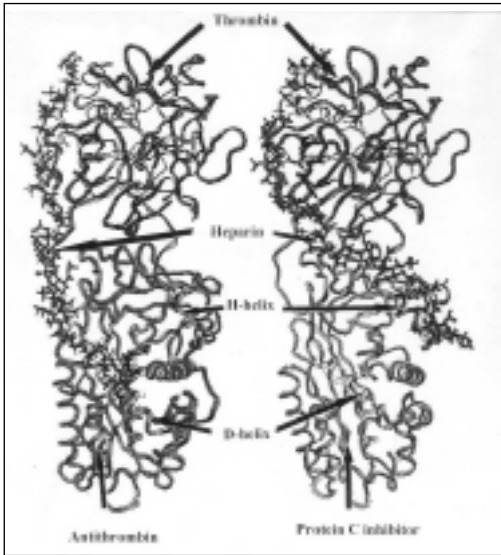
Figure 1: Molecular model of thrombin, antithrombin, heparin complex (left) shows heparin binding to the D-helix on antithrombin. Thrombin, protein C inhibitor, heparin complex shows heparin binding to the H-helix on protein C inhibitor.

MATERIALS AND METHODS

Site-directed mutagenesis of human ATIII cDNA Human AT III cDNA was cloned into the plasmid pGEM between *Eco*RI and *Bam*HI restriction endonuclease sites. Synthetic oligonucleotide primers containing the desired mutations were introduced to the plasmid. Primers were annealed to their homologous sequence on the AT III cDNA at 55˚C. Primers were extended using *Pfu* DNA polymerase in a PCR based reaction. (16 cycles of: 30sec at 95˚C to denature, 60sec at 55˚C to allow primers to anneal, and 9min (2 min/kb) at 68˚C to extend primers) PCR products were digested with *Dpn*I to remove the non-methylated (non-mutagenized) plasmid.

Subcloning of mutagenized cDNA from pGEM into pVL 1392 The plasmid pGEM containing the mutations was digested with restriction endonucleases *Eco*RI and *Bam*HI at 37˚C for one hour. The plasmid pVL 1392 was also digested with the same enzymes under identical conditions. The mutagenized ATIII cDNA was ligated into pVL 1392 using ligase and ATP at 12˚C overnight.

Homologous recombination of mutagenized AT cDNA with Baculogold® DNA using the Baculovirus Expression Vector System® Transfer vector (pVL 1392) containing the AT cDNA containing mutations and Baculoviral DNA were mixed in transfection buffer A. The media was removed from the Sf9 cells and replaced with transfection buffer B. The DNA, in transfection buffer A, was added to the sf9 cells in tranfection buffer B. A precipitate was formed. This induced the cells to undergo endocytosis thus engulfing both the baculoviral DNA and the pVL 1392-AT cDNA. In cells that both DNAs were internalized (co-transfection), homologous recombination can occur. Cells were allowed to grow for 5 days. The media was harvested and then used to infect Hi-5 insect cells. With each successive harvesting the virus was amplified. Pictured below are uninfected Hi 5 cells (left) and infected cells (right).
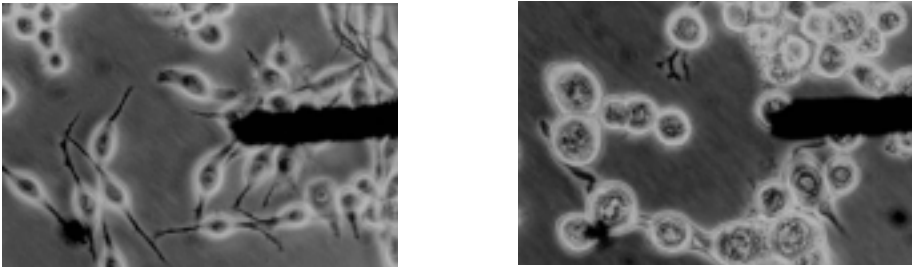
Figure 2: *Tricoplusia ni* (Hi 5) cells Unifected (left) and infected with baculovirus (right)

Purification of recombinant proteins The recombinant proteins were purified out of media via Heparin Affinity Column Chromatography. Recombinant AT binds tightly to Heparin on sepharose beads. All other proteins in media will elute through the column. Tightly bound AT was eluted off the column with 1.5ml of 2M NaCl HNPN (20mM Hepes, 150mM NaCl, 0.1% polyethylene glycol (PEG), 0.02% NaN$_3$ (ph 7.4)) solution. Proteins were dialyzed overnight at 4˚C in HNPN to remove excess salt.

Enzyme Linked Immunosorbant Assay (ELISA) An ELISA was performed to deter-mine concentrations of the recombinant proteins. The purified proteins (100µl) were bound to the wells of a plastic microtiter plate for at least 30 minutes at 37˚C. Wells were then rinsed three time with Tris Buffered Saline with Tween 20 (TBST). A 1% milk/TBST solu-tion to bind any plastic not bound by protein. Blocking also prevents any non-specific bind-ing of antibody to plastic. Rabbit α Human AT III (1˚ Ab) antibody (100 µl) was introduced to the wells and allowed to bind to AT for 30 minutes at 37˚C. The unbound antibody was rinsed out of the wells with TBST. Than Goat α Rabbit antibody conjugated with Alkaline Phosphatase (2˚ Ab) (100µl) was introduced to the each well and allowed to bind to 1˚ Ab for 30 minutes at 37˚C. Para-Nitrophenyl Phosphate was used as a substrate for Alkaline Phosphatase. Amount of AT in each well is proportional to the amount cleaved. This is meas-ured by the absorbance at 405 nm.

Detection of recombinant proteins using an Immunoblot The samples of lysed infected cells and media from the infected cells were electrophoresed on an sodium dodecyl sulfate (SDS) polyacrylamide gel. Samples were prepared in a 1% SDS and 10% β-mercaptoethanol solution to denature the proteins. Proteins on the gel were transferred onto a nitrocellulose membrane using a 300 mA current in a ris Glycine buffer. The proteins were detected in the same manner as the ELISA except Bromo-cloro-indoyl Phosphate and Nitro Blue Tetrazolium (BCIP/NBT) were used as the substrates for Alkaline Phosphatase.

Activity Assay Enzymatic activity assays were performed to study the kinetics of the recombinant proteins. Thrombin (2nM), AT (100nM), and heparin (1µg/ml) were mixed together for 15 seconds. The control contained no AT. Substrate for thrombin was then added along with polybrene, which neutralized heparin. The reaction was allowed to run for 30 min at which time acetic acid (50%) was added to terminate the reaction. Thrombin can only cleave the substrate, thus producing color, when it is not inhibited by AT. Absorbance was read at 405nm.

RESULTS

Mutagenesis Recombinant proteins were successfully made. This was verified using a DNA sequencing gel. The following changes were made:

PCI	^{266}K	T	L	R	K	W	L	K	M	F	K	K	R^{278}
AT	^{302}E	V	L	Q	E	W	L	D$^-$	E$^-$	L	E$^-$	E$^-$	M^{314}
AT-positive (#3)	^{302}E	V	L	Q	E	W	L	K$^+$	K$^+$	L	K$^+$	K$^+$	M^{314}
AT-neutral (#4)	^{302}E	V	L	Q	E	W	L	N	Q	L	Q	Q	M^{314}

Immunoblot An immunoblot was used to show expression of the purified recombinant and wild-type proteins. A sample of human AT was also run on the gel as a control. Analysis of the immunoblot showed that the mutant proteins were being produced at the correct molecular weight (approximately 58 kDa).
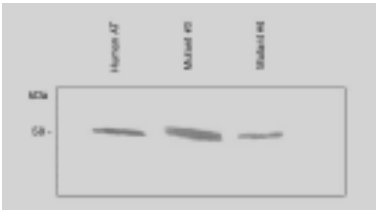

Figure 3: Immunoblot showing the presence of recombinant proteins from media obtained from infected *Tricoplusia* ni insect cells, At-positive (#3), AT-neutral (#4), and human AT

Activity Assay The enzyme assay performed on human AT and recombinant wild-type AT in the presence of heparin present showed lack of activity in the recombinant proteins produced in 1999 (Table 1). Both the human plasma AT and recombinant AT produced from 1997 viral stocks had normal activities. The following kinetic data was found with second order rate constants in $M^{-1}min^{-1}$:

Table 1: Rate Constants for Inhibition of Thrombin ($M^{-1}min^{-1}$)

1997 rAT	3.20E+07
1999 rAT	2.69E+05
Human AT	8.34E+07

DISCUSSION

The negatively charged H-helix in human AT was successfully changed into both a positive and neutral site on separate mutants. These mutations were verified by running a DNA sequencing gel. Tricoplusia ni (Hi 5) insect cells were infected with recombinant Baculovirus containing the mutant AT cDNA. Immunoblot analysis results showed that the mutagenized proteins are being produced by the insect cells at the correct molecular weight. Analysis of activity using an AT specific assay showed that the mutant proteins were inactive. Through further DNA sequencing it was found that the AT cDNA contained a point mutation resulting in an amino acid change of Cysteine[95] to an Argenine. Cysteine[95] is involved in a disulfide bond with Cysteine[21]. This mutation has been found in patients with AT deficiency. Future research plans include correcting the point mutation and studying the kinetic effects of the original H-helix mutations.

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