Small Molecules Binding to Serpins

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Small Molecules Binding to Serpins

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Acknowledgement

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Abbreviations

CHY- chymotrypsin
TRY- trypsin
ACT- $\alpha_1$-antichymotrypsin
AT- $\alpha_1$-antitrypsin or $\alpha_1$-antiprotease inhibitor
Suc-AAPF-pNA- N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-p-nitroanilide
L-BAPNA- $\text{Na}$-Benzoyl- L-arginine 4-nitroanilide hydrochloride
HNE- Human neutrophil elastase
PPE- porcine pancreatic elastase
RCL- reactive center loop
Met- Methionine
His- Histidine
Asp- Aspartate
Ser- Serine
SI- Stoichiometry of Inhibition
TBG- Thyroxine binding globulin
PEDF- Pigment epithelium derived factor
PAI-1- plasminogen activator inhibitor-1
PAI-2- plasminogen activator inhibitor-2
ANS- 2,6-Anilinonaphthalenesulfonic Acid
TNS- 6-(p-Toluidino)-2-napthalenesulfonic acid
Bis-ANS- 4,4'-Dianilino-1,1'-binapthyl-5,5'-disulfonic acid
NBD- nitrobenzoxadiazole
CBG- corticosteroid binding globulin
OVUS- Ovine uterine serpin
PI10- Bomapin
tPa- tissue plasminogen activator
uPa- urokinase-type plasminogen activator
TBS- 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl
Abstract

SMALL MOLECULES BINDING TO SERPINS

By Junaid Haider Afridi, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2006

Dr. Umesh Desai, Associate Professor, Medicinal Chemistry

Serpins are a unique breed of proteins due to their enzymatic mechanism. Two systems were closely monitored during fluorescent binding studies, the ACT-CHY along with the AT:TRY interaction. Four different conformational variants of each system were studied including the native, cleaved, latent and complex forms. Three different fluorescent dyes were used to identify the conformations including ANS, TNS, and bis-ANS. SI studies and protease assays utilizing both Suc-AAPF-pNA and L-BAPNA were instrumental in determining conformations along with gel electrophoresis studies. The hydrophobic dyes bound to the different serpins with varying KD and ΔFmax due to structural variations among the conformers and the complex. Both TNS and bis-ANS gave higher ΔFmax values than ANS. Bis-ANS gave significantly higher ΔFmax values for the ACT:CHY than the other conformations, while also exhibiting relatively low KD value. KD values for the bis-ANS complexes are relatively low when compared to other fluorophores. Bis-ANS is more specific for the AT system than either TNS or ANS. Bis-ANS displays a ΔFmax of 36 fold for the ACT:CHY complex, while TNS displays a 27 fold increase for
AT:TRY system. Modulation studies using bis-ANS to alter the kinetics of latent ACT formation proved unsuccessful, suggesting that fluorescent dyes have little, if any effect on serpin variant formation.
Chapter 1: Introduction

Serpins were first identified as a family of molecules by Hunt and coworkers in 1980, after noticing a primary sequence identity of ~30% among human α1-antitrypsin, human antithrombin, and chicken egg white ovalbumin.¹,² Later, antitrypsin and antithrombin, along with a handful of other similar molecules were found to inhibit proteases containing serine as a catalytic residue, and hence the name serine protease inhibitors (serpins). Many other proteins have now been added to this list, which continues to grow, although many are not inhibitors of serine proteases.³

Today nearly 500 serpins across all species are known to exist. All proteins classified as serpins have a common tertiary structural fold consisting of 3 β-sheets and 8-9 α-helices. Outside of these conserved structural domains the sequence variability is quite large. Most serpins have a molecular weight ranging from 40 to about 65 kDa, while the largest known serpin, C1 inhibitor, has a molecular weight of about 105 kDa.⁴ Some of the unusual serpins include cysteine protease inhibitors, such as squamos cell carcinoma antigen I, and noninhibitory serpins, such as egg white ovalbumin and angiotensin.⁵

The 500 or so serpins have been classified by the HUGO Gene Nomenclature Committee and placed into sixteen different serpin clades based
on their phylogenetic associations. The clades are designated by letters ranging from A through P.\(^6\)

Table 1. Physiological Role of Serpins in the Human Body\(^7\)

<table>
<thead>
<tr>
<th>Serpin</th>
<th>[mg/mL]</th>
<th>Target</th>
<th>Other proteases</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_1)-protease inhibitor</td>
<td>1300</td>
<td>Neutrophil elastase</td>
<td>Trypsin, Pancreatic elastase</td>
<td>Emphysema, cirrhosis</td>
</tr>
<tr>
<td>(\alpha_1)-antichymotrypsin</td>
<td>250</td>
<td>Cathepsin G</td>
<td>Chymotrypsin</td>
<td>Emphysema, Alzheimer's</td>
</tr>
<tr>
<td>Antithrombin</td>
<td>130</td>
<td>Thrombin, Factor Xa</td>
<td>Trypsin, Plasmin, other coagulation proteases</td>
<td>Thrombosis</td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.02</td>
<td>tPa, uPa</td>
<td>Trypsin</td>
<td>Hemorrhage, Thrombosis, metastasis</td>
</tr>
<tr>
<td>Protein C inhibitor</td>
<td>2-5</td>
<td>Activated protein C, uPa, tPA, acrosin Plasmin</td>
<td>Thrombin, Factor Xa, Chymotrypsin</td>
<td></td>
</tr>
<tr>
<td>(\alpha_2)-Antiplasmin</td>
<td>79</td>
<td>Plasmin</td>
<td>Trypsin, Chymotrypsin</td>
<td>Hemorrhage</td>
</tr>
<tr>
<td>C1-Inhibitor</td>
<td>180</td>
<td>C1s, C1r, kallikrein h, Factor X1la</td>
<td>tPa, Factor X1a, Chymotrypsin</td>
<td>Angioedema</td>
</tr>
<tr>
<td>Kallistatin</td>
<td>13.5</td>
<td>Tissue kallikrein</td>
<td>?</td>
<td>Hypo-hypertension</td>
</tr>
<tr>
<td>Heparin cofactor II</td>
<td>80</td>
<td>Thrombin</td>
<td>Chymotrypsin, Cathepsin G</td>
<td>Thrombosis ?</td>
</tr>
<tr>
<td>PAI-2</td>
<td>0.0005</td>
<td>tPa, uPa</td>
<td>?</td>
<td>Cancer (?)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td></td>
<td>Elastase?</td>
<td>Subtilisin Carlsberg</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Taken from Gettins. Four stereo views of α1-antitrypsin, a prototype serpin molecule. The RCL is known as the reactive center loop, and can be seen at the top of the molecule while h denotes α helix, and s denotes β sheet. The important residues are the RCL in yellow, and β sheet C in red.
Human serpins are located on 10 different chromosomes, although a majority of them are located in three clusters. It is interesting to note that within each cluster the serpins belong to the same clade and one explanation for this phenomenon is that many serpins are a result of gene duplication.

Serpins are involved in many important, highly regulated physiological processes including blood coagulation, fibrinolysis, apoptosis, hypertension, hormone transport and inflammation.\(^9,10,11\) In humans 34 serpins have been identified to date and most of them have been well studied and characterized (See Table 1). \(\alpha_1\)-Proteinase inhibitor also called antitrypsin, is quite abundant in our body at normal physiological levels of about 1-2 mg/mL and increases during inflammation.\(^7\) Its main function is to inhibit elastase, which is secreted by human neutrophils during periods of inflammation. Antitrypsin plays an important role in human physiology due to its involvement in multiple disease states and deficiencies. Notably, scientists have been able to link the deficiency of \(\alpha_1\)-antitrypsin to emphysema. Emphysema occurs as a result of the inability to control inflammation, and this situation is further exacerbated by smoking, which leads to oxidation of the P\(_1\) methionine to sulfoxide, making the \(\alpha_1\)-antitrypsin molecule nearly 2000 times less reactive than the thioether.\(^{12}\)

**Native Structure**

The native, or active conformations of serpins have 3 \(\beta\)-sheets along with 9 \(\alpha\)-helices, which corresponds to residues 21-389 of \(\alpha_1\)-antitrypsin (Figure 3).
The three β-sheets contain fourteen strands in all, with β sheet A composed of 5 strands and is the largest as it extends from one end of the serpin skeleton to the other. β-sheet B contains six strands and β-sheet C has three strands. A unique property of the third and fifth strands of β-sheet A is that they are parallel strands, whereas the rest of the strands in β sheets are antiparallel. The native structure also is composed of a reactive center loop (RCL) which is exposed and links β sheet C to β sheet A. The RCL contains anywhere from 22 to 27 amino acid residues labeled P9' to P17, where the P1'-P1 bond denotes the scissile bond. The residues of the RCL define the specificity of serpins, as this area interacts with the potential substrate binding sites of the target protease. The N-terminal (P1-P17) of the RCL must be at least 17 residues long to permit full insertion of residues during formation of cleaved and latent forms.

**Cleaved Structure**

The first structure that was crystallized was of cleaved α1-antitrypsin by Huber et al in 1984. The researchers were actually attempting to crystallize the complexed form of antitrypsin with elastase, but in the process the antitrypsin molecule was cleaved by elastase and the resulting structure was that of cleaved antitrypsin (Figure 2). This cleaved antitrypsin showed strand insertion of the RCL of the antitrypsin into β sheet A between strands 3 and 5. Due to this insertion the ends of what was the scissile bond at P1 were moved about 70 Å apart. This change affects the rest of the serpin molecule, mostly by rearranging
of underlying helices. The process of conversion is quite favorable, resulting in a structure that is stable to denaturation up to about 120°C for the cleaved variant, while studies show that native serpin is stable to about 60°C. Calorimetric measurements for heat released on insertion of the RCL in β-sheet A, and ΔH has a large value of about 60 kcal/mol.

**Latent Structure**

The latent form of α₁-antitrypsin was deduced by Tucker et al. in 1995 simply by placing active native α₁-antitrypsin in a solution of citrate at elevated temperatures for about 12 hours. This variant displayed some similarities to the cleaved form in that the RCL had been inserted in β sheet A as displayed through crystal structures (Figures 1 and 3), although residues of the RCL were still intact in the case of the latent form (Figure 4). These residues provide the return loop towards the top of the molecule in the latent form, while in the cleaved form the C-terminal detached residues make up strand 1 of β sheet C. Formation of the latent structure is favorable for many serpins including α₁-antitrypsin due to its lower energy conformation. This is partially a result of β sheet insertion, although this variant has been shown to be less stable than the cleaved form, and scientists suggest that this is due to the loss in stability by removing a strand from β sheet C.
Figure 1: Cleaved Antichymotrypsin\textsuperscript{17}

Figure 2: Native Antitrypsin\textsuperscript{18}
Figure 3: Latent PAI-1$^{19}$

Figure 4: Antitrypsin-Trypsin Complex$^{20}$
**Serpin-Protease Complex**

Complex formation of serpins had proven elusive to researchers and in 1995, Wright suggested that the RCL of serpins inserts into β sheet A, while still preserving the covalent bond to the protease, which results in a serpin:protease complex with protease at the distal end from the previously uncleaved reactive site loop.\(^{22}\) This suggested a complex structure similar to the cleaved structure, except that the protease remains tightly bound in an irreversible complex. In 2000, Huntington had solved the mystery of the complex structure, and the resulting structure gives insight to how and why the irreversible complex is formed (Figure 5).\(^{9}\) The reaction between serpins and proteases results in a 33% loss in the overall structure for the protease molecule, destabilizing it, while complex formation actually stabilizes the serpin, although a recent study questions this claim. The catalytic triad of the protease in this case is altered such that the serine 195 in trypsin is bound to Met358 of α\(_1\)-antitrypsin, causing the active site area to change conformation, making the catalytic triad less effective in hydrolyzing the acyl bound protease. This conformational change does not disrupt the hydrogen bond between His57 and Asp102, but it does raise the pKa of His57 by about one pH unit.\(^{9}\) This conformational trap prevents catalytic deacylation of the protease, effectively creating a suicide inhibition. This was quite an important discovery because it established a relationship between the conformational change in serpins and reaction with proteinase. Also, reaction with the proteinase causes a dramatic and unique change, one in which
the internal skeleton of the serpin is altered and the proteinase is translocated to the opposite pole of the serpin molecule as evidenced by the cleaved strand located at bottom of serpin. Furthermore, it has also been reported that along with changes in conformation of protease, the hydrolysis of the acyl-enzyme bond is also partially dependent upon the length of the RCL of the serpin.

**Kinetic Mechanism of Serpin Protease Reaction**

The serpin:proteinase reaction occurs in several steps to form either the irreversible inhibitor complex or the cleaved serpin and active enzyme. The 5 steps for serpin reaction as a substrate are: 1) formation of the intitial noncovalent Michaelis complex; 2) active site serine of protease reacting with the P1 peptide of the serpin forming a tetrahedral intermediate; 3) the peptide bond cleaved resulting in a covalent acyl ester intermediate; 4) formation of the second tetrahedral intermediate through addition of water; 5) substrate leaves the active site area. Formation of cleaved serpin occurs as a result of serpins following steps 1 to 5 through completion of reaction, while formation of the irreversible serpin:protease complex follows steps 1 through 3 which may be seen kinetically in Figure 7 and chemically in Figure 8.
Figure 7: Kinetic Mechanism of Serpins

\[
\begin{align*}
E + I & \xrightleftharpoons[k_1]{k_{-1}} EI \\
& \xrightarrow{k_{ac}} E-I \\
& \xrightarrow{k_3} E + I^* \\
& \xrightarrow{k_4} E-I^*
\end{align*}
\]
**Figure 8:** Standard Scheme for serine-protease hydrolysis, shows substrate in an extended conformation in a binding cleft, and key protease residues and interactions. Ser195 of the serine protease is activated by His57 for nucleophilic attack at the carbonyl group of the scissile bond. (a) Acylation proceeds via a tetrahedral intermediate, which is stabilized by favorable interactions between the negatively charged substrate oxygen and amino groups of the protease main chain. (b) Deacylation proceeds via the addition of activated water. P1 and P1' denote the side chains of residues at the scissile bond; R and R' indicate the upstream and downstream fragments, respectively. Taken from Engh et al.25
The first pathway describes the formation of the cleaved substrate serpin and has an overall rate constant of $k_3$. The second pathway describes the acyl intermediate becoming blocked by ineffectiveness of proteinase to complete the proteolysis reaction, and this occurs before step 4. As a result of the loop insertion induced conformational change within the serpin and the resulting distortion of the proteinase active site. The overall rate constant for serpin:protease complex formation is described by $k_4$. Once inactivation of proteinase as a result of complete loop insertion of the RCL has occurred, this results in the proteinase being unable to undergo deacylation. As a result, the serpin is bound irreversibly to the proteinase following the substrate suicide kinetic pathway, $k_4$.

The effectiveness of this reaction is displayed by measuring the $k_5$ value of the complex conversion to cleaved serpin. The $k_5$ value represents deacylation of the serpin:proteinase and studies show that $k_5$ is at least 5-7 orders of magnitude less than $k_3$. The relative proportion of the acyl enzyme complex which is converted to either cleaved serpin or irreversible suicide complex depends on the relationship between $k_3$ and $k_4$. If $k_3 \gg k_4$, the major end product is free enzyme and cleaved serpin, while $k_4 \gg k_3$ gives an irreversible serpin:proteinase complex as major end product. The SI is given by $SI = (k_3 + k_4)/k_4$, and where $k_4$ is much greater than $k_3$ then the SI is $\sim 1$. The key to forming the complex is to have loop insertion occur before the bond is hydrolyzed. Different factors lead to the variation in this ratio which favors one form over another.
Serpin-Ligand Interactions

Determining the Interaction

The ability of serpins to bind to a variety of ligands is useful in studying ways to alter serpin activity. Since serpins play vital roles in our biological systems, studying serpin ligand interactions becomes an invaluable tool in helping to discover serpins’ role in the body, and how altering their activity affects a biological system. The binding of serpins to proteinases in order to inhibit them is the most common serpin interaction studied, and in many cases this interaction may be influenced by a cofactor such as heparin. Serpins in this group include antithrombin, heparin cofactor II, protein C inhibitor, plasminogen-activator inhibitor-1 (PAI-1), protease nexin-1, and kallistatin. Protein ligands bound to serpins may also have an effect on activity and such is the case with PAI-1 binding to vitronectin. Some serpins depend on ligands in order to carry out their function, such as the transport of thyroxine by thyroxine binding globulin (TBG) or the inhibition of blood vessel growth by pigment epithelium derived factor (PEDF).

There are many different techniques to identify and analyze ligand-serpin interactions such as yeast 2-hybrid analysis, spectroscopic techniques, kinetic methods, surface plasmon resonance, and affinity chromatography. The mapping of binding sites on serpins has been studied using chemical modification, site directed mutagenesis, epitope mapping, and crystallography methods. No one method is universally better than the other and many serpin-
ligand interactions have been discovered by multiple techniques to verify the results.

Genetic mapping has allowed us to determine the structure of serpin molecules and their target proteinases. In this case, a simple method to verify whether a ligand may inhibit a serpin is to simply incubate the serpin with ligand, and to measure for activity of the serpin. If serpin activity has been affected, then it is presumed that ligand is interacting with it. Determining a potential ligand may be done using many methods. For those serpin molecules which have been known to bind to non-proteinase ligands for biological reasons, designing and synthesizing novel ligands may be aided by ligand structure. Currently, there is much work being done to improve upon heparin activity creating analogues of its pentasaccharide substructure.

Gel filtration chromatography has proven useful in discovering natural ligands of a serpin. There are many different types of columns, such as size-exclusion, and ion-pair, and different techniques used such as thin layer, and affinity chromatography. This method requires the passage of a biological fluid such as blood plasma thru a column, to which the natural serpin is bound. This elution time is compared with that of free serpin through the column, known as resolution. If there is difference in elution time, it suggests that the serpin is complexed. This is the method which led to the known association between PAI-1 with vitronectin.\textsuperscript{28}
Once a potential ligand has been identified, confirmation of interaction must be determined. Solid phase binding methods may be useful for this purpose. Here, once a potential ligand is identified, it is then immobilized onto a matrix, which is then packed onto a column. A solution containing the target serpin is then eluted through the column. This particular method has been used to establish the binding of several serpins to glycosaminoglycans such as heparin. Examples include antithrombin, heparin cofactor II, protease nexin-1, protein C inhibitor, PAI-1, and kallistatin. The next step would be to measure the affinity of the ligand-serpin interaction. There are several techniques which have proved useful in determining this affinity. One popular technique is solid-phase binding methods. One of the species is immobilized on a solid support, while the other species is added in solution. Once equilibrium is reached, the solid phase is separated from the solution phase and washed. The amount of species bound to the immobilized species may then be quantified. This method has been proven useful for verifying high affinity interactions of a ligand with a serpin, such as protease nexin 1 and C1-inhibitor with collagen. This method is simple, but the major disadvantage is that the immobilized species may be heterogeneous with respect to attachment to the surface. In order to overcome this obstacle, competitive binding approaches may be used. Here the approach is similar to the previous method except that one species is bound to a known ligand, and this complex is progressively dissociated by adding increasing concentrations of the partner species. The difference in total serpin
concentration required to achieve equivalent saturation of the immobilized ligand in the absence and presence of the competitor ligand provides an accurate measure of concentration of the solution serpin-ligand complex independent of the nature of immobilized serpin-ligand interaction.

Quantitating the Interaction

For those situations where one may not be able to immobilize a species onto solid phase, other techniques exist to quantitatively measure the serpin-ligand interactions. One such technique is the use of intrinsic or extrinsic spectroscopic probes to study the interaction. Intrinsic probes are usually found within the aromatic residues of the serpin, which have characteristic near UV absorbance and fluorescence properties, which may be altered as a result of ligand binding. Tryptophan fluorescence has been particularly useful for intrinsic fluorescence measurements. Antithrombin high affinity interactions in the concentration range of 10-20 nM have been studied using fluorescence. It also has proved useful in detecting other serpin-ligand interactions as well. For example, kallistatin, a serpin expressed in endothelial and smooth muscle cells of blood vessels, undergoes a 50% enhancement in intrinsic fluorescence upon binding to heparin. Where the protein ligand possesses intrinsic fluorescence, a difference between the fluorescence of an equimolar mixture of serpin and ligand and the summed fluorescence of free protein and ligand may help indicate
whether the serpin-ligand interaction perturbs the intrinsic fluorescence of one of the interacting components.\textsuperscript{33}

In many cases, the fluorescence energy of the serpin is not affected by the binding of a ligand, and hence an extrinsic fluorophore may be used. These extrinsic fluorophores bind noncovalently to the serpin, which changes the fluorescent characteristics of the fluorophore. Examples of the use of noncovalent fluorescent probes to study serpin-ligand interactions include TNS, ANS, and bis-ANS. ANS has been studied in the particular case of binding to the hormone pocket of the serpin, TBG, with nearly a four fold increase in its fluorescence emission.\textsuperscript{34, 35} Here, binding of hormone displaces the fluorophore and this in turn causes a decrease in fluorescence, a useful technique for quantifying the affinity of the hormone for the serpin. When conducting binding studies with a fluorophore probe, it is important to assess whether the labeling has affected the interaction. This can be done by competitive binding studies in which the unlabeled serpin or ligand is used to compete with the labeled component for binding the species. This was done with the binding of S195A tPA or anhydrotrypsin to recombinant PAI-1 labeled at the P1' reactive center loop residue with the fluorophore, NBD, through an engineered P1' cysteine.\textsuperscript{36} Binding of the labeled PAI-1 to inactive proteases was shown to be weaker then was the unlabeled wild-type PAI-1, by studying the fluorescence changes linked to the displacement of the labeled PAI-1 from its complex with proteinases by unlabeled serpin.\textsuperscript{37} ANS, TNS, and bis-ANS have played an important role in
determining structural changes in the serpin as a result of binding to the ligand, where a ligand bound to serpin causes a change in serpin structure, and this translates into a change in fluorescence. Fluorophores may be used to measure potential binding sites on serpin molecules, because of their small structure, propensity to bind to hydrophobic pockets and the change in fluorescence which may be easily detected. As of the present time, binding studies with these small organic ligands have only been carried out on structural variations of PAI-1.\textsuperscript{27} This interaction may be further studied through the variability in structure and function in serpins to perhaps elucidate potential ligands, which is the focus of this dissertation.
Figure 9: Organic Fluorophores-ANS, TNS, bis-ANS

2,6-Anilinonaphthalenesulfonic Acid (ANS)  6-(p-Toluidino)-2-napthalenesulfonic acid (TNS)

4,4'-Dianilino-1,1'-binapthyl-5,5'-disulfonic acid (bis-ANS)
Table 2: Selected serpins and their known proteinase and nonproteinase ligands

<table>
<thead>
<tr>
<th>Serpin</th>
<th>Nonproteinase ligands</th>
<th>Proteinase target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin Xa,</td>
<td>Heparin, heparan sulfate</td>
<td>Thrombin, factor Xa</td>
</tr>
<tr>
<td>Antichymotrypsin</td>
<td>DNA, Aβ1–42 peptide</td>
<td>Cathepsin G</td>
</tr>
<tr>
<td>α, Proteinase inhibitor</td>
<td></td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>C1-inhibitor</td>
<td>Heparin, collagen</td>
<td>C1s</td>
</tr>
<tr>
<td>CBG</td>
<td>Corticosteroids</td>
<td>−</td>
</tr>
<tr>
<td>Headpin</td>
<td></td>
<td>Lysosomal cathepsin (?)</td>
</tr>
<tr>
<td>Heparin cofactor factor II</td>
<td>Heparin, dermatan sulfate</td>
<td>Thrombin</td>
</tr>
<tr>
<td>Hsp47</td>
<td>Collagen</td>
<td>−</td>
</tr>
<tr>
<td>Kallistatin</td>
<td>Heparin</td>
<td>Tissue kallikrein</td>
</tr>
<tr>
<td>Maspin</td>
<td>Collagen</td>
<td>−</td>
</tr>
<tr>
<td>MENT</td>
<td>DNA</td>
<td>Nuclear cysteine proteinase (?)</td>
</tr>
<tr>
<td>OVUS</td>
<td>Activin</td>
<td>−</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Heparin, heparan sulfate, vitronectin, thrombin, aPC</td>
<td>tPA, uPA,</td>
</tr>
<tr>
<td>PEDF</td>
<td>Heparin, collagen</td>
<td>−</td>
</tr>
<tr>
<td>PI10</td>
<td></td>
<td>Nuclear proteinase (?)</td>
</tr>
<tr>
<td>Protein C Inhibitor</td>
<td>Heparin, retinoic acid</td>
<td>aPC, thrombin, acrosin, uPA</td>
</tr>
<tr>
<td>Protease nexin-1</td>
<td>Heparin, collagen</td>
<td>Thrombin, uPA</td>
</tr>
<tr>
<td>TBG</td>
<td>Thyroxine, triiodothyronine</td>
<td>−</td>
</tr>
<tr>
<td>ZPI</td>
<td>Protein Z</td>
<td>Factor Xa</td>
</tr>
</tbody>
</table>
The interaction between serpins and ligands from a mechanistic point of view gives a better look at the potential effect of ligand binding. Because serpins go through a branched pathway suicide substrate mechanism, which permits formation of either the cleaved non-complexed or cleaved complexed form, the effect of the ligand on serpin function may be determined. This can be done by titrating a fixed concentration of enzyme with increasing concentrations of serpin in the presence and absence of ligand. After this reaction has reached
completion, the residual enzyme activity is determined by monitoring the initial rate of hydrolysis utilizing a suitable chromogenic substrate. The stoichiometry can then be determined from the x-intercept of a plot of residual activity vs. the ratio of serpin/protease concentration. If the ligand has affected the serpin pathway, then either or both rate constants of mechanistic pathways will be altered. This pattern was seen for heparin reaction in the presence of antithrombin, thrombin and factor Xa. Measurements of the change in $K_{obs}$ can help to provide a means of measuring the affinity of serpin-ligand interaction. Measuring change in $K_{obs}$ for serpin:protease as a function of ligand concentration should be nearly equivalent to a binding curve for serpin-ligand interaction. This has been seen extensively with antithrombin-binding heparin pentasaccharide on the kinetics of antithrombin reaction with its target proteinases.

More complex kinetic perturbations may also be studied. One common observation is that the ligand produces a bell shaped dependence of $K_{obs}$ on ligand concentration in which the rate constant increases then plateaus at a peak, and then decreases. This effect is usually seen in the case of heparin, where the ligand binds both the serpin and proteinase. The initial increase is the result of formation of protein-ligand binary complex and when the ternary complex reacts with proteinase. The decrease at higher ligand concentrations reflects progressive blocking of ternary complex formation due to excess free ligand competing with the serpin-ligand complex for proteinase.
In order to measure the specificity of the ligand serpin interaction, substitution of important residues on the serpin which interact with ligand or changing ligand structure may be done. In the case of glycosaminoglycans, the specificity was determined by immobilizing serpin on a gel while passing different structural variants of ligand through the gel. This procedure was done for low-affinity and high-affinity binding heparin. Here, the high-affinity heparin had a specific sequence giving it the high affinity binding. Mutagenesis is also a powerful tool for determining potential binding site on a serpin. In this approach, an expression system and a screening assay are required to identify any mutants with defective ligand binding but which are normally folded. Once the region has been identified, specific residues important in binding may be determined by mutagenesis and analysis of interaction. An exosite on antithrombin in the RCL region was shown to be quite important in factor Xa binding. This procedure has helped to establish common binding sites on antithrombin, heparin cofactor II, PAI-1, and protease nexin-1 in interaction with glycosaminoglycans.

The most preferred method of mapping a ligand binding site on a serpin molecule and establishing whether a ligand has the capacity to induce conformational changes on the serpin is to solve the crystal structure of the serpin in the free, or unreacted state and complexed to ligand by X-ray diffraction. Here, ligand induced changes in antithrombin caused by heparin of both the binding site and the reactive center loop were easily evident by the free and complexed structures. In the case of protein C inhibitor in complex with two
known ligands, retinoic acid and heparin, the structure of the complex has yet to be determined, although the free structure suggests binding sites of the hormone and heparin. The structure of the ternary bridging complex of heparin with the serpin and two proteinases, thrombin, and activated protein C, show that the serpin and proteinase would have to bind to opposite sides of a common site on heparin for the serpin RCL to interact with proteinase active site.$^{54}$

**Heparin Pentasaccharide-Antithrombin**

Among the many ligands studied to date, several successfully inhibit serpins, and they are listed in the following table. Of particular interest is heparin, due to its wide range of targets within the serpin family, and which is a prime example of how ligand serpin interactions can yield therapeutic results. Heparin is a naturally occurring heterogeneous sugar polysaccharide found in the human body, and it plays a vital role in anticoagulation.$^{7}$ The polymer form is comprised of 5 different monosaccharide units in varying amounts and is very acidic due to sulfate and carboxylic acid groups. Heparin pentasaccharide, which is the form of heparin used to bind to serpins, contains five unique sugar units denoted by DEFGH and has a molecular weight of about 1500 g/mol. Heparin activity with antithrombin has proven to be especially important for therapeutic reasons. There are two distinct mechanisms for heparin-activated antithrombin inhibition of factor Xa and thrombin. The interaction of high-affinity heparin, or pentasaccharide H5, expels the partially inserted RCL residues
altering the conformation of the P1-P10 reactive center and exposing an exosite in antithrombin (Fig. 5). This sequence of events is called the conformational activation of antithrombin. The altered RCL in heparin-antithrombin co-complex is easier recognized by factor Xa and this results in accelerated cleavage of the P1-P10 bond and quick formation of the covalent inhibited complex (E*-AT* in Fig. 2). As a result of activation of antithrombin, factor Xa inhibition is increased sufficiently. Thrombin inhibition, in contrast, is accelerated only twofold through the conformational activation mechanism (see Fig. 4). The main effect of heparin in accelerating thrombin inhibition is due to a bridging mechanism. Tight binding of antithrombin to the H5 sequence in full length heparin is followed by the binding of thrombin to the same chain at non-specific sites to form an antithrombin–heparin–thrombin ternary complex (Fig. 5). Thrombin then diffuses along the polyanionic chain to encounter the inhibitor resulting in a ~2,000-fold acceleration in inhibition under physiological conditions. A saccharide length of about 18 residues is needed to concurrently bind thrombin and antithrombin for the accelerated inhibition. Thus, while sequence-specific H5 is necessary for tight binding of heparin chains, the H5 alone cannot potentiate antithrombin inhibition of thrombin. Here again the ligand in this case was reacted in the presence of both antithrombin and thrombin where heparin concentration was varied and a bell-shaped dependence of acceleration on the concentration of heparin chains, characteristic of a bridging mechanism, was observed.
Another serpin-ligand interaction of interest is that of plasminogen activator inhibitor-1 (PAI-1) and vitronectin. PAI-1 is a member of a group of proteins known to inhibit plasminogen activators. When fibrinolysis occurs, tissue plasminogen activator (tPa) converts protein plasminogen into plasmin. As a result, plasmin then degrades fibrin along with providing for localized protease activity including ovulation, and cell differentiation. PAI-1 is the main inhibitor of tPA, and controls the production of plasmin, and as a result controls fibrinolysis. Vitronectin is a large glycoprotein with a molecular weight of about 75 kDa, and it also plays an important physiological role. It has been known to target antithrombin-thrombin complex, along with glycosaminoglycans including heparin playing a role in coagulation. It's major role is debatable but it is present in tissues and helps to promote cell adhesion. Interaction of vitronectin with PAI-1 is not known to inhibit the activity of active PAI-1, as vitronectin bound
to PAI-1 is bound to the lower half of the serpin, in particular binding to residues on helix F, and strands 3 and 5 of β sheet A.

**Thyroxine-TBG; Corticosteroid-CBG**

Globulins including both thyroxine binding globulin (TBG), and corticosteroid binding globulin (CBG) bind to smaller organic molecules, and give us more insight into small molecule influence on serpin activity. Globulins play a major role in the immune system as they are carriers of antibodies. Thyroxine is a derivative of the amino acid tyrosine, and contains 4 iodine atoms. It is also the major hormone secreted by the thyroid gland. Nearly all of the thyroxine in the body is bound to TBG. TBG’s most vital role in the body is as a carrier of thyroxine throughout the body so that it may help regulate metabolism and physical development. The structure of TBG and the binding site of thyroxine is still unknown to this date. Corticosteroids are a family of compounds including cortisol which is an adrenal hormone. There have been nearly a hundred synthetic derivatives of cortisol, which are being sold as drugs. Their main role is to block allergen and inflammatory activity by blocking prostoglandins, yielding powerful anti-inflammatory activity. The structure of CBG has been solved, although as is the case with TBG, the binding site of corticosteroids is unknown.
Retinoic Acid-Protein C Inhibitor

Retinoic acid interaction with Protein C inhibitor is also of interest, due to similarities in size of retinoic acid and the fluorophore ANS. Protein C inhibitor's physiological role is to inhibit activated protein C and thrombin through its interaction with heparin, playing a dual, competing role in the anticoagulation pathway. Heparin is believed to bind to protein C inhibitor at the H helix, which is at the backside of the molecule directly behind \( \beta \) sheet B. Retinoic acid is a organic derivative of vitamin A found in the epidermal and dermal layers of the skin, whose function it is to aid in the development and maintenance of bones and skin. The consequence of an interaction of retinoic acid with protein C inhibitor is unknown although it is thought to be a delivery protein in the same way that TBG delivers thyroxine throughout the body. Retinoic acid is hypothesized to bind in an area near helix A of protein C inhibitor, although the three dimensional structure of protein C inhibitor is not yet known.

In 2001, a group of researchers documented an association between bis-ANS and PAI-1. The structures of the conformations of PAI-1 show great variability between the active and the cleaved/latent forms. In the native structure, there is a large hydrophobic pocket located adjacent to strand 1 of \( \beta \) sheet A, and between both helix D and A, which is missing in the variants due to strand insertion. When the conformational variants were tested for fluorescent activity in the presence of bis-ANS, the native conformation displayed an intensity much greater than for cleaved, latent, or PAI-1 complexed to urokinase.
type plasminogen activator (uPA) as can be seen from graph 1. Since the hydrophobic region is unique to the native form, and this area consists of mainly hydrophobic residues, it is hypothesized that bis-ANS binds in this particular region, which can be seen on Figure 10. The residues implicated in binding to bis-ANS are shown in red, while those residues involved in vitronectin binding are shown in green.
Figure 12: Fluorescence of 4 mM bis-ANS with selective conformations of PAI-1

![Fluorescence spectrum of 4 mM bis-ANS with selective conformations of PAI-1.]

- Active PAI-1
- Latent PAI-1
- Reactive center-cleaved PAI-1
- uPA-PAI-1 complex
- Buffer control

Figure 13: Binding site of bis-ANS and vitronectin on PAI-1.

![Diagram showing the binding site of bis-ANS and vitronectin on PAI-1.]
Chapter 2: Methods

Proteins and Stock Solutions

Bovine $\alpha_1$-antitrypsin (MW 57kDa) purchased from Sigma was in lyophilized powder form. 5 mg of antitrypsin was dissolved in 965 $\mu$L of 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl (TBS) to give a stock solution of 0.1 mM. Smaller volumes were aliquoted into 1.5 mL tubes at a concentration of 10 $\mu$M and kept at 4°C for storage. Bovine trypsin (MW 25 kDa) was also purchased from Sigma. Twenty mg. of the trypsin stock was diluted with 1 mM HCl buffer to give a concentration of 43.5 $\mu$M in solution and was stored at -20°C.

Human $\alpha_1$-antichymotrypsin (ACT) was purchased in powder form from Calbiochem, while bovine $\alpha$-chymotrypsin (CHY) was purchased from Sigma. One mg ACT was dissolved in 615 $\mu$L TBS, to give a stock concentration of 25 $\mu$M, while 10 mg CHY was dissolved in 10 mL 1 mM HCl solution to give a stock concentration of 40 $\mu$M, which was subsequently diluted to desired concentrations for creating ACT-CHY complex.

The substrate used for the CHY assay was Suc-AAPF-pNA (MW=625 g/mol), which was bought from Sigma in lyophilized powder form. Substrate is initially dissolved in 1 ml DMSO to 3 mM, which was diluted to 2 mM with
additional TBS. On reaction with CHY the para-nitroaniline group is cleaved from peptide and displays a yellow color. This is measured by UV/Vis at 405 nm.

The substrate used in the trypsin assay was Nα-benzoyl-L-arginine p-nitroanilide (L-BAPNA), which was purchased from Sigma in lyophilized powder form. L-BAPNA has a molecular weight of 435 Da. L-BAPNA substrate was dissolved in 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl to give a 1 mM stock solution. The 1 mM L-BAPNA was placed at 4°C fridge for storage.

**Km and Vmax of CHY and TRY**

The Km and Vmax determinations for Suc-AAPF-pNa reaction with CHY was carried out by reacting 10 nM CHY with various concentrations of substrate ranging from 0 to 100 μM in 1 mL TBS solution. The slopes of the reaction were recorded and the slope value were plotted against substrate concentrations. A curve fit based on the equation, \( y = \frac{V_{\text{max}} \times x}{K_d + x} \), was fit to the data points on the graph.

The Km determination for the substrate was determined by aliquoting 10 μL of the 43.5 μM stock solution into 900 μL TBS. 200 nM trypsin was titrated with L-BAPNA ranging from 0 to 500 μM. The Km was determined by fitting the data to the previous equation.
The stoichiometry of inhibition (SI)

10 nM CHY was incubated with a range of concentrations of ACT from 0 to 40 nM in 100 μL TBS overnight at 25°C. Next, the solution was diluted with 850 μL of Tris-HCl buffer and 50 μM Suc-AAPF-pNA substrate was added to give a total volume of 1 mL. The absorbance was recorded for 3 min. at 405 nm. The values of the slope measurements were noted and a plot of slope versus [ACT]₀/[CHY]₀ was prepared. From the plot, a linear regression of the data to obtain the point at which the line crosses the x axis gave the stoichiometry of inhibition. The reaction of trypsin with antitrypsin is monitored with L-BAPNA substrate. 200 nM trypsin is reacted with 0 to 500 nM antitrypsin in 1 mL TBS with 250 μM L-BAPNA.

Preparation of cleaved serpin

ACT

Cleaved ACT was prepared by diluting stock ACT solution into 1 mL TBS to a concentration of 1 μM. Human neutrophil elastase (HNE), purchased from Calbiochem, was added to give a final concentration of 20 nM or 1/50th the concentration of ACT. With the concentration of HNE being only a fraction of ACT, complex formation does not occur. The solution was incubated at 37°C for thirty minutes and every 5 minutes a sample was checked for residual CHY activity using hydrolysis of 50 μM Suc-AAPF-pNA substrate. The solution was
then stored at -20°C, when the residual activity reached 3%, or 97% cleaved ACT was formed.

AT

10 μM native antitrypsin solution is diluted to 1 μM in 1 mL TBS. 40 μM porcine pancreatic elastase (PPE), purchased from Sigma, was added to give a final concentration of 1 μM. The ratio of serpin to proteinase was 1:1. This solution was incubated at room temperature for 30 minutes. Samples were taken every 10 minutes to check for activity by adding trypsin and 250 μM L-BAPNA substrate, and after 90 minutes the activity of trypsin was ~96%, suggesting 96% cleaved antitrypsin.

Preparation of latent serpin

ACT

Formation of latent ACT was done by diluting a stock solution of native ACT to 1 μM in 1 mL TBS and then placed in 37°C warm water bath.72 The activity of CHY against ACT was repeatedly tested using hydrolysis of 50 μM Suc-AAPF-pNA substrate. In about 4 week's time, the enzymatic activity reduces to only about 5% suggesting ~95% latent or polymerized form.

AT

The latent form of α1-antitrypsin was prepared as previously reported.73 The 10 μM stock solution of antitrypsin was diluted 10 fold with TBS to yield a final concentration of 1 μM antitrypsin. The solution was incubated at 67°C for
approximately 24 hrs. Samples were taken periodically to check for activity by adding trypsin and 250 μM L-BAPNA substrate, and after 24 hrs, antitrypsin activity was about 3%, suggesting 97% latent or polymerized form. Antitrypsin forms the latent complex at higher temperatures, but it is between 65-68°C, that formation of latent AT is maximized and polymerization is minimized. After 24 hours, the latent AT solution was stored at -20°C until further analysis.

**Preparation of Serpin-Protease Complex**

Native ACT and CHY, each at a concentration of 1 μM, were mixed in a 1 mL solution of TBS. The mixture was incubated for thirty minutes. Using the CHY assay previously discussed, the residual CHY activity was repeatedly measured. Following completion of reaction, the residual CHY activity is expected to be near zero. After verification that complex formation is complete, sample is stored at -20°C.

Formation of AT-TRY complex was very similar to ACT-CHY complex. Equimolar ratios of native AT and TRY were mixed at 1 μM in 1 mL TBS. The mixture was incubated for 30 minutes, and assayed for activity using the TRY assay previously described. Residual TRY activity is expected to be near zero. After verification that complex formation was complete, sample was stored at -20°C.
**Preparation of stock solutions of fluorophores**

For this study, three hydrophobic aromatic fluorescent dyes have been chosen: 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS), 2-(p-toluidinyl)naphthalene-6-sulfonic acid (TNS), and (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS). ANS was purchased from Sigma as a lyophilized powder (>97% purity, HPLC). TNS was also purchased from Sigma in lyophilized powder form (>97% purity, HPLC), while bis-ANS was purchased from Molecular Probes in lyophilized form also (>95% purity, HPLC). 260 mg ANS was dissolved in 1 mL DMSO then diluted with 1.4 mL TBS to give a 34 mM stock solution. This solution was further diluted to give final concentration of 3.4 mM. Twenty mg of TNS was dissolved in 75/25 ethanol (mL)/DMSO (mL) mixture to 1 mL to give a 6.8 mM solution. Once fully dissolved, it was further diluted with 30 mL of TBS to give a 1.7 mM stock solution. The bis-ANS was previously diluted in 2 mL deionized water. The absorbance of the solution was detected and measured at 395 nm, and using Beer’s Law, the concentration was measured to be about 2.4 mM, with extinction coefficient of 23,000 cm⁻¹ at 395 nm. This solution was further diluted to 1.25 mM with 1.84 mL TBS.

**Determination of emission wavelength of fluorophores**

In a 3 mL acrylic cuvette, 25 μL of 3.4 mM ANS was pipetted into a 1 mL solution of TBS to give a 85 nM solution. The slits for the emission and excitation
monochromators were .5 mm. The excitation wavelength was set at 404 nm, and emission was recorded from 425 to 600 nm. TNS spectrum analysis was performed in a manner similar to ANS. For TNS, the excitation wavelength was 330 nm, while emission was recorded from 350 to 600 nm. The saturation concentration of TNS is about 20 μM. For bis-ANS the excitation wavelength was 425 nm, while emission is recorded from 440 to 600 nm. The maximum fluorescence was recorded for each fluorophore and used as emission wavelengths for titration procedures.

**Titration Procedures**

Three control samples were titrated: 1) fluorophore in buffer solution, 2) protease (chymotrypsin/trypsin) and fluorophore in buffer solution, and 3) porcine pancreatic elastase (PPE) and fluorophore in buffer solution. Using a PEG coated methacrylate cuvette, stock fluorophore 1 μL at a time up to 50 μM was added to 1.1 mL TBS, to measure the background fluorescence for all following titrations. The protease plus fluorophore titration was done to ensure no change in fluorescence occurs due to protease only. PPE and fluorophore control was measured due to the high concentration in solution during titration. For both protease and PPE 6.8 mL of 40 μM protease was diluted into 1.1 mL TBS to 250 nM. Fluorophore was added 1 μL at a time up to 50 μM. The results determined that protease and PPE had insignificant effect on the fluorescent activity of fluorophore.
Gel Electrophoresis Analysis

10% acrylamide gels were made with the following procedure: 2.5 mL of 40% acrylamide, and 2.5 mL of pH 8.8 separating gel buffer were added to a 5.0 mL water solution. Solution was degassed and 10 μL TEMED and 50 μL ammonium persulfate were added to mixture, mixed and added to gel plates. For stacking layer, 2.5 mL 40% acrylamide, 2.5 mL pH 6.8 stacking gel buffer were added to 5.0 mL water. Solution was degassed and 10 μL TEMED, and 50 μL of ammonium persulfate were added to mixture, mixed and added to the separating layer.

For samples, all conformational variants were at a concentration of 5 μM in 25 μL TBS. Six μL of 6X SDS buffer was added directly to stock, mixed and then placed in gel. The protein standard was purchased from Bio-Rad and was diluted 1:31 by adding 1 μL to 24 μL TBS, and 6 μL 6X SDS buffer. Gels were run until dye passed through gel. Gels were stained in Coomassie staining solution for two hours, and then destained in 88/5/7% volume mixture of water/methanol/acetic acid overnight.

Modeling of Protein Surface

The trypsin-antitrypsin complex protein structure (1EOZ) was retrieved through PDB and analyzed using Sybyl. Formal charges were added using the Gasteiger-Huckel method. A surface scan was conducted using the fast Connolly method, and the hydrophobic potential was then mapped, where red
designated the most lipophilic residue, while blue designated the most hydrophilic.
Chapter 3

Objectives

There were three main objectives of this study:

1) To determine whether the different conformational states of serpins can be differentiated with hydrophobic dyes using fluorescence spectroscopy.

2) To determine whether a hydrophobic binding pocket exists in selected conformational forms of serpins.

3) To determine whether the proteinase inhibitory activity of serpins can be modulated with hydrophobic fluorophores.

The first objective is probably the most important because it attempts to develop an easy protocol for assessing the state of serpins. Knowing what form of serpin is in a given sample using fluorescence could be a good analytical tool compared to enzyme inhibition studies, due to its sensitivity and simplicity. Objectives 2 and 3 depend directly on the development of the first objective. The second objective points to a more fundamental principle. Many proteins have hydrophobic pockets in their complex 3D structure, and this study will help to determine if the same is true for serpin molecules. Along with this, the study will help to determine whether there is a specific
hydrophobic pocket within the serpin molecule, which one or more of the fluorophores may bind to, and whether this pocket is conserved among the different conformational structures of serpins. While this study is being undertaken, favorable interactions will also be sought out as an increase in fluorescence intensity results from more hydrophobic character of interaction. This interaction will help to determine if a hydrophobic binding pocket exists, where it is, and if it is conserved among serpin subtypes such as antichymotrypsin and antitrypsin. Finally, objective 3 attempts to connect the hydrophobic pocket to serpin activity. Organic fluorophores will be exposed to native serpin during conversion into the latent form, to determine whether the fluorophores have any affect on the rate of this conversion, or modulation. Modulating the activity of serpin is a useful tool, as it might affect the in-vivo activity of serpins in humans. Many disease states involve the interactions of serpins with other molecules and with themselves, and manipulating their activity could possibility have therapeutic potential.
Chapter 4: Results

Background

The initial goal of this work was to find a simple technique to distinguish the three conformational forms of a serpin and the serpin:proteinase complex. Serpins exist in a variety of conformations. A structural change within the serpin molecule affects the inhibitory activity of the serpin. These structural changes might be monitored by using the change in fluorescence of extrinsic fluorophores, which bind noncovalently to hydrophobic domains on the surface of protein molecules. Changes in the structure of a protein affect either quantum yield or affinity of these extrinsic fluorophores, which may be monitored. This has been shown in studies with ANS and ovalbumin. Using mutant forms of ovalbumin that displayed loop insertion properties, ANS was found to bind primarily when loop insertion occurred. The binding of ANS resulted in an increase in fluorescence of the complex suggesting additional ANS molecules bound on loop insertion. Those mutants which do not loop insert showed a decrease in fluorescence signal, suggesting the release of ANS on proteolytic cleavage of the RCL. A similar study was also conducted by Egelund et al. in which the fluorescence decreases on proteolytic cleavage of the RCL of PAI-1. Structural
and binding studies were also conducted on PAI-1 binding to bis-ANS, a dimer of ANS. In addition to binding to bis-ANS, the fluorescence binding studies show binding to three different serpin variants along with urokinase type plasminogen activator. See Graph 1, Chapter 1.

Another study proposed the existence of a hydrophobic binding region within a mutated form of PAI-1, and designed a ligand based on these findings. A study conducted in 1998 by Lomas et al. described the formation of a canonical inhibitory conformation of a mutant of antitrypsin. The study went further to reveal a difference in structure between the wild-type and mutant forms, and in particular a hydrophobic region within the wild-type between strand 2 of β sheet A and helices D and E. The study suggested that the hydrophobic binding pocket may be exploited for creating ligands which do not allow the loop to insert.

The purpose behind the use of α1-antichymotrypsin and chymotrypsin as model system stems from it's importance in Alzheimer's disease, wherein amyloid beta (Aβ) peptide occurs in insoluble plaques along with other molecules, including α-antichymotrypsin. In-vitro studies show that Aβ binds to ACT. α1-Antitrypsin was also studied due to Lomas's work in detecting a potential hydrophobic binding pocket in serpins. Binding studies conducted on the serpins/proteases will help to understand the characteristics of the variants and help to probe the differences among them. Using the hydrophobic fluorescent probes ANS, TNS, and bis-ANS binding studies will enable
determination of favorable binding pockets within the structures. Using this knowledge, analogues of one or more of the fluorescent probes may be synthesized in order to target one or more of the protein structures. These analogues may then be used to design a small molecule marker, which may affect protease inhibitor. Furthermore, future molecular modeling studies will help to determine where on the protein these fluorescent molecules bind.
Km and Vmax of (CHY-Suc-AAPF-pNA) and (TRY-L-BAPNA) Assays

CHY-Suc-AAPF-pNA Assay

Suc-AAPF-pNa is a blocked polypeptide ester with a phenylalanine side chain, which is a preferred substrate for chymotrypsin. 1.25 μL of 280 μM CHY was assayed for activity against different concentrations of Suc-AAPF-pNA substrate ranging from 0 to 100 μM, in a 1 mL TBS. The slopes were calculated, and the values shown on the following graph, where the x value is concentration of substrate and y value is the slope of the reaction curve. The Km value was 46 ± 4 μM, while Vmax was 108 ± 5 mAbs/min.

Figure 1. CHY Assay w/substrate

10 nM CHY assay with Suc-AAPF-pNA
TRY-L-BAPNA Assay

Trypsin catalyzes hydrolysis of amides and esters of basic amino acids, such as the p-nitroanilide of benzoyl arginine (BAPNA). In the assay, 250 nM trypsin is reacted with various concentrations of 1 mM L-BAPNA ranging from 0 to 500 μM in 1 mL TBS. The following graph was plotted, and x/y values are the same as for the CHY-substrate reaction. The Km values were 233 ± 15 μM, while the Vmax was calculated to be 39 ± 1 mAbs/min.

Figure 2: TRY Assay w/substrate

250 nM TRY assay with L-BAPNA

![Graph showing the assay with L-BAPNA](image)
Determining Activity of Stock Serpin Solutions (SI)

$\alpha_1$-Antichymotrypsin

ACT activity was checked against CHY solution by taking 9 samples of 2.5 μM ACT varying from 0 to 40 μL (0 to 100 nM) and mixing each with 8 μL 10 μM CHY, 50 μM Suc-AAPF-pNA and TBS to 1 mL. The change in absorbance is measured spectrophotometrically and the fractional chymotrypsin activity at each concentration of ACT is plotted vs. the nominal ratio of ACT/CHY. A straight line regression slope is used to determine the molar ratio of ACT required to inhibit CHY. The linear decrease in CHY activity as ACT concentration increases is due to formation of inhibition complex. The stoichiometry of inhibition obtained from this plot is 1:1.6. The stock solution of ACT is 63% active and in native form.

Figure 3: SI determination for ACT
$\alpha_1$-Antitrypsin (AT)

The AT SI experiments are conducted in a similar manner to ACT as outlined in the methods section. AT activity against TRY solution was measured by taking 6 samples of 25 mM AT varying from 0 to 20 mL (0 to 450 nM) and mixing each with 5 mL 40 mM TRY, 200 mM L-BAPNA and TBS to 1 mL. The slope of the curves for trypsin catalyzed hydrolysis of L-BAPNA was measured spectrophotometrically, by measuring absorbance at 405 nm. In reaction with native AT, TRY was mixed into buffer solution, incubated overnight, diluted to 1 mL, and then reacted with TRY substrate to check for residual activity. The SI for AT is 2.3:1, consistent with the supposed activity listed by Sigma.

Figure 4: SI determination for AT
Preparation of Serpin:Protease complex

ACT-CHY

Activity of CHY decreased when equimolar concentrations (1 μM) of ACT and CHY are allowed to react for at least 10 minutes. Here, complex formation occurs and due to presence of CHY, no additional CHY is added. A sample of ACT:CHY was checked for inhibitor activity as a function of time, by taking a 10 μL sample at various times and mixing it with 965 μL of TBS, and 50 μM Suc-AAPF-pNA, and following the increase in absorbance at 405 nm spectrophotometrically. The activity of CHY should decrease due to formation of ACT:CHY complex and after about 10 minutes, the activity of CHY leveled off. Thus, ACT and CHY at 1 μM were incubated for at least 10 minutes to ensure the formation of ACT-CHY complex in subsequent experiments.

Figure 5: ACT-CHY reaction w/substrate
AT-TRY

Initially, 1 μM of TRY solution was mixed with 1 μM active AT in 1 mL TBS for 15 minutes. A sample of the AT:TRY solution was checked for trypsin activity every 3 minutes by taking a 25 μL sample of 5μM AT:TRY, mixing it with 200 μM L-BAPNA and 865 μL of TBS and measuring the absorbance change at 405 nm spectrophotometrically. Activity against L-BAPNA substrate decreases exponentially to about 3% after 12 minutes. Thus, AT-TRY at 5 μM was incubated for at least 12 minutes to ensure formation of AT-TRY complex in subsequent experiments.

Figure 6: AT-TRY reaction w/substrate
Preparation of Cleaved Serpin

ACT

One μM cleaved ACT is prepared as previously described by digestion with HNE. The concentration of ACT in the preparation was 1 μM, while the concentration of HNE was 20 nM. Samples of cleaved ACT was checked for activity against CHY solution, by taking a 10 μL sample and mixing it with 1 μM CHY, 50 μM Suc-AAPF-pNA and 964 μL of TBS. This mixture was then monitored at 405 nm for CHY activity spectrophotometrically, and a linear increase in absorption indicating first order kinetics. Samples were taken every 15 minutes until CHY activity was greater than 95%, indicating almost complete loss of ACT activity (98% cleaved ACT after 90 minutes).

Figure 7: Cleaved ACT reaction w/substrate
AT

The cleaved conformation is formed using 1 μM PPE and incubating at room temperature with 1 μM AT as discussed in methods. Every 5 minutes a sample of AT was checked for activity against trypsin by hydrolysis of L-BAPNA, by taking a 25 μL sample of 5 μM cleaved AT and mixing it with 12.5 μL 10 μM TRY in 1 mM HCL, 200 μM L-BAPNA and 863 μL of TBS. The linear absorption change at 405 nm was measured spectrophotometrically. After about 30 minutes TRY activity was 96%, indicating 96% cleavage of antitrypsin.

Figure 8: Cleaved AT reaction w/substrate
Preparation of Latent Serpin

ACT

Stock solution of 1 μM native ACT was placed in 1 mL TBS at 37°C in a warm water bath. Over 4 week's time, the solution was checked for activity by adding 10 nM with 50 μM Suc-AAPF-pNA in 1 mL TBS, and measuring absorbance at 405 nm using UV/Vis. A linear increase was seen in each measurement, and this indicated that the reaction followed first order kinetics. After 4 weeks time, the solution showed nearly 95% residual CHY activity, indicating that only 5% of the ACT remained active.

Figure 9: Latent ACT reaction w/substrate
Using the 10 μM stock solution of antitrypsin, 500 μL was pipetted into a tube containing 1 mL of tris HCl pH 7.5 buffer to a concentration of 5 μM antitrypsin, which was then placed in hot water bath at 67°C for optimal latent formation. Periodically 25 μl samples were measured for TRY activity, by mixing it with 12.5 μL 10 μM TRY in 1 mM HCL, 200 μM L-BAPNA and 863 μL of TBS. This mixture was then analyzed for activity as described. Each measurement displayed linear increase in slope at 405 nm, characteristic of first order kinetics. After a period of 24 hours, the residual TRY activity was 95%, suggesting 95% latent antitrypsin. Polymer formation may have occurred even though reaction took place at 67°C, which is optimal for latent formation.

Figure 10: Latent AT reaction w/substrate
SI results

Cleaved ACT

The SI test was measured to ensure formation of cleaved ACT using concentration ratios ranging from 0 to 6:1 of cleaved ACT:CHY and extrapolating a linear slope to the x-axis. In particular, 10 nM of CHY solution is incubated with concentrations of cleaved ACT ranging from 0 to 60 nM in a 1 mL solution containing TBS and 50 μM Suc-AAPF-pNA. Hydrolysis was measured spectrophotometrically, and the CHY activity displayed as a function of the ratio of (cleaved ACT)/(CHY) concentrations. Even at a 6:1 ratio of cleaved ACT to CHY, activity is at or near 100%. Using a linear regression line the slope of SI is positive, and the value of the SI of cleaved ACT: CHY reaction is undefined.

Figure 10: SI Determination of Cleaved ACT
Latent ACT

To determine the latent ACT SI, samples of latent ACT at concentrations from 0-60 nm were incubated with 10 nM CHY in 1 mL TBS and 50 μM SucAAPF-pNA. The hydrolysis of substrate was measured 405 nm using UV/Vis spectrophotometer, and the %CHY activity was plotted as a function of the ratio of (latent ACT)/(CHY) concentrations. The graph displays the ratio of latent ACT to CHY, and the slope decreases slightly giving an SI ratio of 65:1. Even at a molar ratio of 6:1, the activity of CHY was unaffected by latent ACT. The ACT has about 1.5% activity, a decrease of 60% from the native SI.

Figure 11: SI determination of Latent ACT
ACT-CHY complex

ACT-CHY complex was tested for its activity using CHY to ensure that all of the CHY was in complex. Again, the procedure was similar to other conformational variants of ACT. A 10 nM CHY solution was incubated with concentrations of ACT-CHY complex ranging from 0 to 60 nM in 1 mL TBS and CHY substrate, Suc-AAPF-pNA. The solution was then analyzed for hydrolysis activity at 405 nm using UV/Vis spectrophotometer, and the CHY activity was displayed as a function of the ratio of (ACT-CHY)/(CHY) concentrations. From the above graph, at high molar ratios of 6 to 1, the CHY activity is at or near 100%. Any free CHY in solution would have displayed increased activity with increasing ratios. This is not the case as the activity remains near the control CHY activity.

Figure 11: SI Determination of ACT-CHY complex
Cleaved AT

Varying concentrations of cleaved AT ranging from 0 nM to 1.2 μM are mixed with 200 nM native TRY in 1 mL TBS, along with 200 μM L-BAPNA and the absorbance was measured at 405 nm. The SI of cleaved AT was 60:1.

Figure 13: SI Determination of Cleaved AT
Latent AT

The AT SI experiments are conducted in a similar manner to ACT SI experiments as outlined in the methods section and the results are strikingly similar. Varying concentrations of latent AT ranging from 0 nM to 1.2 μM with 200 nM native TRY in 1 mL TBS with 200 μM L-BAPNA were assayed. Absorbance change at 405 nm showed linear increase. The ratio of [latentAT]/[TRY] ranged from 0 to 6. At a ratio of 6 to 1, the TRY activity remains high, near 100%. The SI ratio for latent AT was about 65:1, indicating that 65 moles of latent AT are required to inhibit 1 mole of native TRY.

Figure 12: SI Determination of Latent AT
**AT-TRY complex**

Varying concentrations of AT-TRY complex ranging from 0 nM to 1.2 μM are mixed with 200 nM native TRY in 1 mL TBS, along with 200 μM L-BAPNA. The linear increase in absorbance was measured at 405 nm. The SI for AT-TRY was 56:1.

Figure 14: SI Determination of AT-TRY complex
UV and Fluorescence Spectrum

The fluorescence spectra were measured before and after titrations for accuracy. It is important to choose a point where the absorbance values are quite low (at or near 0.1 mAbs/min) to ensure that the inner filter effect is not affecting titration studies. The following graph displays the emission spectrum for ANS from which an excitation wavelength of 425 nm was chosen. The emission spectrum was recorded from 450 to 600 nm and an emission value of 526 nm, which corresponds to a maxima peak. TNS was excited at 330 nm, and emission recorded from 365 to 500 nm. Bis-ANS was excited at 395 nm while emission was recorded from 425 to 500 nm.

Figure 15 and 16: ANS
Fluorescence Spectrum of 60µM ANS; Excitation = 404 nM, Excitation = 440-600 nM

UV Spectrum of 20 µM TNS in buffer
Fluorescence Emission Spectra of 20 μM TNS; Excitation = 330 nM, Emission = 365-500 nM

UV Spectrum of bis-ANS at 20μM in buffer with α1-ACT conformers
Fluorescence Emission Spectrum of bis-ANS with $\alpha_1$-ACT conformers; Excitation = 395 nm Emission = 450 to 550 nm

SDS-PAGE

SDS allows proteins to be separated according to their molecular weight. The latent and native forms of serpins should have a very similar molecular weight, while the cleaved form will be slightly less due to missing strands at the C terminus after cleavage of the P1-P1' scissile bond. SDS is a highly negatively charged molecule, which denatures the protein by coating the surface with multiple negative charges, and therefore creates a uniform shape for all proteins that it comes in contact with. Using an 10% acrylamide gel and low molecular weight protein standards bought from Bio Rad, gel electrophoresis can be done
with known molecular weights. The protein standards include proteins ranging from 15-95 kDa, covering the desired range of 50-70 kDa. For all conformations of antichymotrypsin, 25 μl of 5 μM sample is mixed with 5 μl SDS and placed in gels after heating. The following gel shows all conformations of antichymotrypsin, along with protein standards.

Figure 23: SDS PAGE Gel of Serpins

The protein standards include albumin at a MW of 66,000 and is the second from top mark. Using this as a guide, the different conformations of ACT can be seen right across from it in rows 2-5. Antichymotrypsin has a molecular weight range from 65 to 68 kDa, so it should appear close to albumin standard, and does so. Cleaved ACT is lower than either the native or latent forms, due to
loss of 3 kDa in cleavage. Antitrypsin shows up at about the 55 kDa range, and both latent and native conformations are as predicted. Cleaved antitrypsin should show up at a slightly lower molecular weight, yet it shows up at the same level of both the latent and native versions. More importantly, the complexed versions of both ACT:CHY and AT:TRY fail to show up on any of the gel-studies conducted. Unfortunately, substrate activity is the only measure of determining the presence of complex.

Titrations

Introduction

ANS, TNS, and bis-ANS are fluorophores that bind to hydrophobic pockets of proteins. The fluorophores fluoresce, and when bound to proteins the intensity of fluorescence light emitted changes. Any variation in the fluorescence arises from a change in the environment of the fluorophore. This change is generally an increase on association and a decrease on dissociation of the fluorophore. This change may be interpreted as monitoring the binding.

In this particular study ANS, TNS, and bis-ANS have been used to titrate all four conformations of ACT and AT. In order to accurately measure any association that a fluorophore may have with either molecule, a background study must be conducted every time the machine is used to check for lamp stability, to subtract from the titrations, and most importantly to simply make sure the fluorophore is active, stable and working properly. All titrations were
conducted in 1100 μL Tris-HCL pH 7.5, with fluorophore stock solution added in
1 and 2 μL increments while measuring the emission. Fluorescence data is
interpreted simply as the difference of intensity of each titration minus the initial
reading. The data is converted to percentage change in fluorescence data to
make the readings more useful and to standardize between experiments. Over
time, the lamp used to conduct runs weakens so therefore the raw data results
may actually vary by a great deal. The curve fit used is the $K_A$ equation as given,

$$ F = \left( \frac{\Delta F_{\text{max}}}{0.5} \right) \left( \left[ L \right]_0 + K_D + [R]_0 \right) - \left( \left[ L \right]_0 + K_D + [R]_0 \right)^2 - \sqrt{4 \times \left[ L \right]_0 \times [R]_0}, $$

where $f$ is fit to $y$. Here $K_D = [R]_0[L]_0/[RL]$, where $[R]_0$ is the receptor (serpin,
serpin: protease) concentration in this case is 250 nM and $[L]_0$ is the ligand
concentration which changes for each titration point.

**ANS, TNS, and bis-ANS backgrounds**

The fluorophore is added in small increment to 1100 μLTBS. Mixing
homogeneity is important for an accurate measurement. There should be a linear
increase in emission with increasing fluorophore concentration.

**Native ACT**

Results of native ACT binding experiments with ANS are displayed on the
following graph (Fig. 24). The graph shows that the native ACT fluorescence
intensity increases by about 100% in reaction with ANS with a $\Delta F_{\text{max}}$ value at 95
± 2%. $K_D$ values for the association are 7.5 ± 0.8 μM suggesting that ANS binds
with quite high affinity. At a value of about 7.5 μM, the native form binds with about twice the affinity of the complex and nearly 8 times greater affinity than the latent form. This value is lower than the latent form and complex while greater than the cleaved form. The ΔFmax and KD values show that native ACT binds tighter than latent and cleaved ACT, while the ΔFmax values are also higher than for the cleaved and latent form.

Native ACT binding experiments with TNS display greater affinity of the fluorophore for the native protein as is the pattern with all serpin variants. The KD values are 12.1 ± 1.0 μM, similar to all other serpin variants in reaction with TNS. Native ACT has the RCL exposed while both latent and cleaved ACT have loop inserted into β-sheet A. This difference in structure may explain the differences in the KD and ΔFmax.

**Cleaved ACT**

Cleaved ACT is taken from stock and placed in 1100 μL TBS. Titration studies are carried out using 1 μL ANS aliquots at a time reaching a maximum concentration of ANS at 80 μM, while protein concentration is maintained at 250 nM. It is important to note that there is a residue of human neutrophil elastase within the cleaved ACT sample, but since the sample ratio is 1:100 of ACT to HNE, a concentration of 2.5 nM is negligible in the solution of target serpin and ligand. The results in figure 24 show a small relative increase in the fluorescence of ANS. The KD value for ACT cleaved-ANS complex was found to be 9.1 ± 3.0
μM by fitting the data to the quadratic binding equation. The ΔFmax values are 8.8 ± 0.8%. Cleaved ACT shows very little change from the background in fluorescence (about 9%).

Binding studies with TNS are conducted in a similar manner (figure 25). Since TNS is a more sensitive fluorophore than ANS, titrations are conducted 1 μL at a time to a final concentration of 20 μM in a protein solution of 250nM. Results with TNS are differentiated from ANS by a greater increase in relative fluorescence. The point of saturation is at a concentration 4 times less than that of ACT. Although the K_D values are almost identical to the ANS reaction at 9.1 ± 1.5 μM, the ΔFmax values are more than 10 times greater at 109 ± 8%.

Results from the bis-ANS saturation show the same trend as that for ANS and TNS. Fluorescence intensity measurements show that the cleaved ACT shows the lowest change of all the serpin variants. The K_D values for cleaved ACT were recorded but are quite suspect due to irregularity in the fluorescence results. There was quite a bit of variation in the three experiments. The K_D from averaging the three experiments come out to 31 ± .03 μM, which is 3 times higher than either ANS or TNS. ΔFmax values were found to be 166 ± 1%.

Latent ACT

Structurally the latent form differs from the cleaved by an extra loop which returns to the top of the serpin. In addition cleaved ACT has an extra strand in β
sheet C. These two structural differences might cause variations in fluorescence with different fluorophores.

Fluorescence binding experiments with ANS show some interesting results. The $K_D$ values for latent ACT are $51.2 \pm 6.6 \mu M$, which is about 4 times higher than the other three variants. This leads to some interesting questions about the structure of the latent form. The uniqueness of its structure must play some part in the weak binding of ANS. Latent ACT has an extra loop return which probably affects the ability of ANS to bind to the scaffold of ACT as it does on the native and cleaved versions. The $\Delta F_{\text{max}}$ is quite high at $328 \pm 19\%$, an increase of over 3 times the initial background study.

Latent ACT displayed the highest relative change in fluorescence with TNS. In terms of fluorescence TNS would serve as the best option for identifying the latent ACT form by fluorescence methods. $K_D$ values are quite high according to the curve fit equation, which means that although TNS does not bind with the same affinity to the latent form as the other variants, the $\Delta F_{\text{max}}$ for latent ACT is higher with TNS than the other fluorophores. The change in fluorescence is greater than 700%. $K_D$ values for reaction with TNS are $12.9 \pm 0.6 \mu M$, which are in line with affinities of other serpin variants of ACT for TNS. The $\Delta F_{\text{max}}$ values for this reaction are $765 \pm 15\%$, a value nearly 2 times greater than the next highest value. This suggests that the latent form has more hydrophobic pockets to which TNS associates than other variants.
Bis-ANS shows the second highest change in fluorescence. Its relative change in fluorescence is nearly 400 times the initial fluorescence reading. The \( K_D \) values are \( 10.7 \pm 4.2 \, \mu\text{M} \), displaying an affinity lower than either the native form or the complex. The \( \Delta F_{\text{max}} \) values are \( 494 \pm 11\% \), a 500\% increase from the initial background studies. This value pales in comparison to the complex reaction with bis-ANS, but it is nearly equivalent to the native form. From these experiments, it is safe to assume that both TNS and bis-ANS are suitable fluorophores for detecting the presence of the latent form.

**ACT-CHY complex**

The results of relative fluorescence intensity change show that the complex displays the greatest increase of all four structures on binding fluorophore. The fluorescence intensity change of nearly 33\% relative to the two native active protein structures, and is not due to binding to CHY. ANS titrations with \( \alpha \)-chymotrypsin showed minimal fluorescence binding.

In binding experiments conducted in the presence of ANS fluorophore, the ACT-CHY complex displayed a \( K_D \) value of \( 15.4 \pm 1.9 \, \mu\text{M} \), a value only slightly different from the other serpin variants. The \( \Delta F_{\text{max}} \) value for this reaction was \( 286 \pm 4\% \), which is on the higher end of the spectrum, although lower than the latent form.

In reaction with TNS, the results were similar to the native form. The \( K_D \) values for complex reaction with TNS are \( 11.2 \pm 1.4 \, \mu\text{M} \), while the \( \Delta F_{\text{max}} \) values
are $369 \pm 22\%$. The $\Delta F_{\text{max}}$ values for both the TNS and ANS reaction show that complex interaction with these fluorophores consistently creates a noticeable change in the intensity of fluorescence.

Complex binding with bis-ANS gave a $K_D$ value of $4.5 \pm 0.8 \mu\text{M}$, lower than complex reaction with other fluorophores, making bis-ANS the most sensitive fluorophore in reaction with ACT-CHY complex (figure 26). The $\Delta F_{\text{max}}$ value at $3569 \pm 249\%$ shows the greatest increase in intensity. In fact the $\Delta F_{\text{max}}$ value is nearly 36 times greater than the background, a noticeable difference in intensity. Bis-ANS is clearly a good candidate for further study and analysis in its interaction with the ACT-CHY complex.
Figure 24

**ANS titration of ACT- Native, Cleaved, Latent and ACT:CHY complex**

<table>
<thead>
<tr>
<th>Serpin Conformation</th>
<th>K(_D) ((\mu)M)</th>
<th>(\Delta F_{\text{max}}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT(_L)</td>
<td>51.2 ± 6.6</td>
<td>328 ± 19</td>
</tr>
<tr>
<td>ACT(_C)</td>
<td>9.1 ± 3.0</td>
<td>N/A</td>
</tr>
<tr>
<td>ACT(_N)</td>
<td>7.5 ± 0.8</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>ACT-CHY</td>
<td>15.4 ± 0.9</td>
<td>286 ± 4</td>
</tr>
</tbody>
</table>
Figure 25

TNS titration of ACT- Native, Cleaved, Latent and ACT:CHY complex

<table>
<thead>
<tr>
<th>Serpin Conformation</th>
<th>$K_D$ (µM)</th>
<th>$\Delta F_{\text{max}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT_L</td>
<td>12.9 ± 0.6</td>
<td>765 ± 17</td>
</tr>
<tr>
<td>ACT_C</td>
<td>9.1 ± 1.5</td>
<td>109 ± 8</td>
</tr>
<tr>
<td>ACT_N</td>
<td>12.1 ± 1.0</td>
<td>339 ± 15</td>
</tr>
<tr>
<td>ACT-CHY</td>
<td>11.2 ± 1.4</td>
<td>369 ± 20</td>
</tr>
</tbody>
</table>
Figure 26

B-ANS titration of ACT- Native, Cleaved, Latent and ACT:CHY

<table>
<thead>
<tr>
<th>Serpin Conformation</th>
<th>$K_D$ (µM)</th>
<th>$\Delta F_{max}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT$_L$</td>
<td>10.7 ± 4.2</td>
<td>494 ± 11</td>
</tr>
<tr>
<td>ACT$_C$</td>
<td>.31 ± 0.3</td>
<td>166 ± 1</td>
</tr>
<tr>
<td>ACT$_N$</td>
<td>2.5 ± 0.4</td>
<td>547 ± 29</td>
</tr>
<tr>
<td>ACT-CHY</td>
<td>4.5 ± 0.8</td>
<td>3569 ± 248</td>
</tr>
</tbody>
</table>
Native AT

Native AT shares a structural conformation similar to native ACT in the fact that it has an exposed RCL. The RCL of native AT differs from native ACT by four amino acids, and its P1 group is methionine as opposed to arginine for native ACT. As a result of this similarity, binding studies should show a pattern similar to ACT, as is seen for the cleaved and latent forms.

Binding studies conducted with ANS reveal that the $K_D$ value is $10.4 \pm 0.7 \mu M$ and the $\Delta F_{\text{max}}$ value is $185 \pm 3\%$. Native antitrypsin shows greater affinity for ANS than either the latent or complex form. In reaction with both TNS and bis-ANS, native AT has higher $\Delta F_{\text{max}}$ values than the cleaved form pointing to greater association of fluorophores to the native form versus the cleaved. $K_D$ values calculated for TNS and bis-ANS are $9.3 \pm 0.6 \mu M$, and $2.8 \pm 0.3 \mu M$, while $\Delta F_{\text{max}}$ values are $976 \pm 26\%$, and $573 \pm 17\%$. Affinity values point to the fact that bis-ANS is the best fluorophore for binding to the native form, while $\Delta F_{\text{max}}$ values point to TNS as being the best in determining increase in fluorescence.

Cleaved AT

Cleaved AT is formed by reaction with porcine pancreatic elastase (PPE). The procedure calls for an equimolar ratio of elastase to serpin thus there is an appreciable amount of PPE remaining in the reaction thus pancreatic elastase is also titrated to correct for background.
Results of the cleaved AT binding to ANS is shown on figure 27. Cleaved AT shows the lowest relative increase in fluorescence intensity as also noted with cleaved ACT. The $K_D$ value was found to be $10.3 \pm 0.9 \, \mu M$, while the $\Delta F_{\text{max}}$ value was $153 \pm 4\%$.

Both TNS and bis-ANS binding results for cleaved AT are similar to results for ANS in that relative fluorescence changes are the lowest of all the conformations. The $K_D$ values for TNS bound to cleaved AT are $11.6 \pm 1.4 \, \mu M$, while $\Delta F_{\text{max}}$ values are $743 \pm 45\%$. For bis-ANS the results are $1.2 \pm 0.1 \, \mu M$ for $K_D$, while the $\Delta F_{\text{max}}$ is $401 \pm 6\%$. There is not much difference in affinity between ANS and TNS, while bis-ANS at $1.2 \, \mu M$ binds at about 10 times less concentration.

Latent AT

Binding studies to ANS, TNS, and bis-ANS are seen on figures 23-25. Latent AT shows some similarity to latent ACT in its high $K_D$ values. According to the curve fit results, bis-ANS would be the best candidate for binding studies with latent AT. The results for binding to ANS are $17.3 \pm 0.8 \, \mu M$ for $K_D$, while the $\Delta F_{\text{max}}$ values are $247 \pm 4$. TNS give a $K_D$ value of $7.9 \pm 0.5 \, \mu M$ and $\Delta F_{\text{max}}$ values of $686 \pm 18\%$. In reaction with bis-ANS, the values are $3.6 \pm 0.4 \, \mu M$ for $K_D$ and $869 \pm 32\%$ for $\Delta F_{\text{max}}$. Bis-ANS shows the greatest change in fluorescence according to the graphs, and the $\Delta F_{\text{max}}$ values are highest for bis-
ANS, while $K_D$ values suggest that TNS has the greatest affinity for latent AT of the fluorophores studied.

**AT-TRY complex**

The AT-TRY complex shows similar results in that $\Delta F_{\text{max}}$ values for the complex are higher for all three fluorophores bound to it. $K_D$ values for ANS, TNS, and bis-ANS are $21.3 \pm 0.6 \, \mu M$, $10.5 \pm 0.4 \, \mu M$, $1.6 \pm 0.1 \, \mu M$ respectively. Here, it is quite evident that bis-ANS shows the greatest binding affinity to the complex. Although AT-TRY shows a high increase in affinity with bis-ANS at about 10 fold, complex association with TNS is 26 fold over initial readings, as evident by $\Delta F_{\text{max}}$ values for the complex. The $\Delta F_{\text{max}}$ values are $618 \pm 7$, $2634 \pm 46$, $910 \pm 13$ for ANS, TNS and bis-ANS respectively. Here it is important to point out that the complex shows $\Delta F_{\text{max}}$ values higher than any other form of serpin whereas in the ACT system the latent form had slightly higher $\Delta F_{\text{max}}$ values in association with both ANS and TNS, than the complex. It is difficult to pinpoint exactly why the complexes display such differences except to point out that the two serpin:proteinase complexes are structurally different and these differences point to variability in exosite binding structures, where the fluorophores may bind.
Figure 27

**ANS titration of AT- Native, Cleaved, Latent and AT:TRY complex**

<table>
<thead>
<tr>
<th>Serpin Conformation</th>
<th>$K_D$ (µM)</th>
<th>$\Delta F_{\text{max}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{TL}$</td>
<td>17.3 ± 0.8</td>
<td>247 ± 4</td>
</tr>
<tr>
<td>$A_{TC}$</td>
<td>10.3 ± 0.9</td>
<td>153 ± 4</td>
</tr>
<tr>
<td>$A_{TN}$</td>
<td>10.4 ± 0.7</td>
<td>185 ± 3</td>
</tr>
<tr>
<td>AT-TRY</td>
<td>21.3 ± 0.6</td>
<td>618 ± 7</td>
</tr>
</tbody>
</table>
Figure 28

TNS titration of AT- Native, Cleaved, Latent and AT:TRY complex

<table>
<thead>
<tr>
<th>Serpin Conformation</th>
<th>K_D (µM)</th>
<th>ΔF_{max} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATL</td>
<td>7.9 ± 0.5</td>
<td>686 ± 18</td>
</tr>
<tr>
<td>AT_C</td>
<td>11.6 ± 1.4</td>
<td>743 ± 45</td>
</tr>
<tr>
<td>AT_N</td>
<td>9.3 ± 0.6</td>
<td>976 ± 26</td>
</tr>
<tr>
<td>AT-TRY</td>
<td>10.5 ± 0.4</td>
<td>2634 ± 46</td>
</tr>
</tbody>
</table>
Figure 29

B-ANS titration of AT- Native, Cleaved, Latent and AT:TRY complex

<table>
<thead>
<tr>
<th>Serpin Conformation</th>
<th>$K_D$ (µM)</th>
<th>$\Delta F_{\text{max}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{TL}$</td>
<td>$3.6 \pm 0.4$</td>
<td>$869 \pm 32$</td>
</tr>
<tr>
<td>$A_{TC}$</td>
<td>$1.2 \pm 0.1$</td>
<td>$401 \pm 6$</td>
</tr>
<tr>
<td>$A_{TN}$</td>
<td>$2.8 \pm 0.3$</td>
<td>$573 \pm 17$</td>
</tr>
<tr>
<td>AT-TRY</td>
<td>$1.6 \pm 0.1$</td>
<td>$910 \pm 13$</td>
</tr>
</tbody>
</table>
**Overall Results**

$K_D$ values of the serpin variants indicate some important things. If the ligand is unchanged while the receptor environment is varied, then the results may be analyzed in terms of the most favorable receptor. Table 30 shows that there seems to be no consistent pattern. ANS binds all serpin variants with about the same affinity, as is also the case for TNS and bis-ANS. Looking down each column where the serpin variant is kept constant and the ligand changes, one can notice a trend. The $K_D$ values decrease as the ligand changes from ANS to TNS to bis-ANS.

**Figure 30. Overall $K_D$ values**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$ACT_L$</th>
<th>$ACT_C$</th>
<th>$ACT_N$</th>
<th>$ACT$-CHY</th>
<th>$AT_L$</th>
<th>$AT_C$</th>
<th>$AT_N$</th>
<th>AT-TRY</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS</td>
<td>51.2 ± 6.6</td>
<td>9.1 ± 3.0</td>
<td>7.5 ± 0.8</td>
<td>15.4 ± 1.9</td>
<td>17.3 ± 0.8</td>
<td>10.3 ± 0.9</td>
<td>10.4 ± 0.7</td>
<td>21.3 ± 0.6</td>
</tr>
<tr>
<td>TNS</td>
<td>12.9 ± 0.6</td>
<td>9.1 ± 1.5</td>
<td>12.1 ± 1.0</td>
<td>11.2 ± 1.4</td>
<td>7.9 ± 0.5</td>
<td>11.6 ± 1.4</td>
<td>9.3 ± 0.6</td>
<td>10.5 ± 0.4</td>
</tr>
<tr>
<td>Bis-ANS</td>
<td>10.7 ± 4.2</td>
<td>N/A</td>
<td>2.5 ± 0.4</td>
<td>4.5 ± 0.8</td>
<td>3.6 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>2.8 ± 0.3</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>
The $\Delta F_{\text{max}}$ values are more useful. Across each row where the ligand environment is kept constant and the receptor is variable, few patterns are discernible. First, the cleaved form of the serpin shows the least change in $\Delta F_{\text{max}}$ values suggesting that the hydrophobic binding pocket within this structure does not associate well with all three ligands. Both latent and complex forms of the serpin show consistently higher $\Delta F_{\text{max}}$ values than either the native or cleaved. This may be interpreted as both variants associating to a higher degree than the native and cleaved variants of the serpins, and pointing to a binding site with greater hydrophobic character or more number of hydrophobic sites. More importantly, differentiation between the conformational variants is possible due to the differences in $\Delta F_{\text{max}}$ values. Within each column conclusions are more difficult to make. On average, both bis-ANS and TNS associate to a greater degree than ANS, due to higher $\Delta F_{\text{max}}$ values.

**Modulation of Enzyme Activity**

It's known that pH, concentration of ions, and temperature have an adverse affect on enzyme activity. For example it is known that chymotrypsin
and trypsin perform optimally at pH 8.5, and at 37°C. The presence of other molecules on enzyme activity is seen in the human body and this keeps enzymatic activity under physiological control. The purpose of performing a modulation study with organochemical ligands and serpin molecules is to deduce whether these molecules affect serpin activity. If these ligands do affect activity, then this knowledge can be used to further study the interaction. More importantly, effects on enzyme activity may be exploited for medicinal purposes, especially in disease states where serpin molecules are involved.

The particular study that was undertaken to check for modulation of serpin activity by bis-ANS involved setting up the experimental procedure for generating the latent form of serpin molecules. 100 μl of 2 μM antichymotrypsin is allowed to incubate with 10 μM bis-ANS. A similar sample of antichymotrypsin by itself is incubated as a control measure. 10 μl samples are measured at 0, 1, 3, 5, 8 and 24 hours to measure for residual chymotrypsin activity. The results of this experiment are displayed on the following graph (figure 32). The graph shows that there is little if any influence of bis-ANS on the activity of native antichymotrypsin conversion to the latent form. At all the time points the residual chymotrypsin activity of both the sample with bis-ANS and the sample w/bis-ANS are within 5% of each other. From the results of the graph one can conclude that bis-ANS will have an inconsequential effect on the activity of antichymotrypsin.
Hydrophobic Binding Domains in Serpin Conformations

After determining our results, the next step is to model the complex, and look for potential hydrophobic pockets which may exist within the external 3-dimensional structure. The following figure (33) shows the scaffold of antitrypsin-trypsin complex (1EOZ), and below it is a topological surface rendering of the complex, using SYBYL. Looking at the hydrophobic potential surface scan (figure 34), the colors at the top or brown areas represent the most hydrophobic potential, whereas the colors at the bottom represent hydrophilic potential. Just from viewing the face of 50% of the molecule there are several potential pockets where bis-ANS may bind. There are two pockets to the right of strands 1 and 2.
of β sheet A, and to the left of helix E which are similar to pockets implicated in vitronectin:PAI-1 potential binding.

Figure 33: 3-D structure of AT-TRY complex

Figure 34: Hydrophobic potential map of AT-TRY complex
Chapter 5: Conclusions

Returning to our objectives we may be able to put in perspective the results from our study. The first objective deals with differentiation of the conformations of serpins using fluorescence spectroscopy. From the results the first objective has been met, and this is due to the unique values for each of the conformations when fluorophore is bound.

Both the antitrypsin and antichymotrypsin systems display some common and unique results. Both the AT and ACT show that the TNS and bis-ANS fluorophore are overall more sensitive in detecting the presence of both serpin systems, while ANS is the least sensitive, and this is apparent from looking at the $\Delta F_{\text{max}}$ values. The $K_D$ values do not show any consistent pattern amongst the respective serpin systems.

In terms of unique results, bis-ANS reaction with ACT has the greatest affinity of the three fluorophores for ACT. In particular, bis-ANS showed elevated association to the ACT-CHY complex, while bis-ANS did not show similar results for the AT system. For the AT system, the AT-TRY complex displayed elevated association with TNS fluorophore. This difference in results can be exploited through detailed study of the external structure of both complexes. ACT and AT share 45% homology, therefore differences in complex structure are likely.
From the binding studies of the ACT system there are a few clear conclusions. Both TNS and bis-ANS give higher $\Delta$Fmax values than ANS. Concentrations used for ANS are about 10 times lower for titrations. Bis-ANS gives significantly higher $\Delta$Fmax values for the ACT: CHY than the other conformations, while also exhibiting relatively low $K_D$ values averaging 5 $\mu$M.

From the binding studies of the AT system a few conclusions may also be made. Both TNS and bis-ANS also give higher $\Delta$Fmax values than ANS. $K_D$ values for the bis-ANS titration are relatively low when compared to other dyes. Bis-ANS has greater affinity for the AT system than does either TNS or ANS. $\Delta$Fmax values for TNS titration of AT: TRY complex are significantly higher than other structural conformers of AT.

Fluorescent dyes bind tightly to hydrophobic pockets within the protein structure. Bis ANS displays an increase in fluorescence 36 times higher for ACT:CHY complex than for buffer solution alone, and TNS 27 times higher for the AT:TRY than buffer alone. With the data given, designing analogues to probe the structure of the serpin: protease complex to determine the degree of interaction and ability to control the kinetics of this interaction are possible.

The second objective, modulation of serpin activity by fluorophores, showed that exposing bis-ANS to native ACT during it's conversion to latent ACT had no significant effect on its activity. Therefore, it is concluded that fluorophores, in particular bis-ANS, does not modulate the transition between native and latent serpin structures.
The third objective, to determine whether there is a conserved binding site on serpins, was addressed and reveals numerous hydrophobic pockets which may bind small organic fluorophores such as ANS. Determining whether binding occurs to a specific pocket might prove difficult, due to differences in serpin structure and the large number of potential binding pockets. In order to study this, further tests must be conducted including modeling and mapping of the external structure of serpins.

As a final note, formation of the conformations yielded some problems. First, the formation of latent conformation was verified using assay, and procedure which limits polymer formation, but the possibility of polymers forming in solution still remains. The gel electrophoresis studies show presence of the latent confirmation, but this could just as easily be polymers, as they are known to form from ionic bonding, which would be broken in the presence of SDS. The second issue has to do with the results of the gel electrophoresis experiments. Unfortunately, formation of complex was not verifiable through this method, although procedures for complex formation were adhered to, and assay experiments validated complex formation.
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