A PEPTIDE MODEL FOR THE HEPARIN BINDING SITE ON ANTITHROMBIN III

by

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ABSTRACT

Heparin is a highly heterogeneous, sulfated proteoglycan, the saccharide portion of which is widely used in the treatment of thrombosis. The anticoagulant activity of heparin results from its activation of several serine protease inhibitors (serpins), including ATIII, which are critical for the regulation of the blood clotting cascade. ATIII, a 432 amino acid glycoprotein, undergoes a conformational change upon binding to a specific rare sequence within heparin. Binding results in a 1000-fold increase in the rate at which ATIII neutralizes the proteases thrombin and factor Xa. A crystal structure of ATIII has not been reported. Because of the size of the protein, spectroscopic studies of ATIII bound to heparin have yielded little detailed structural information.

This thesis project focused on elucidating the molecular details of the interaction between heparin and Antithrombin III (ATIII). One general model for this interaction is that a specific sequence in heparin recognizes and/or stabilizes a non-native conformation at one or more sites at the ATIII surface. The approach taken was based on the idea that peptides can be used to model flexible regions on a proteins surface.

The heparin binding site on ATIII is assumed to involve a region on the surface of the protein having a high density of basic residues. Residues 123-139 of ATIII have been implicated in heparin binding by a variety of methods. Three peptides were prepared whose sequences are derived from this region of ATIII.

ATIII 123-139:	acetyl-FAKLNCRLYRKANKSSK-amide	
ATIII N135 GlcNAc	acetyl-FAKLNCRLYRKA**N**KSSK-amide	**N** = Asn(GlcNAc)
ATIII Random:	acetyl-FKAKNCRLYRAKSSNLK-amide	

The sequence of peptide ATIII 123-139 reflects that of the native protein. It has been proposed that the sequence of 123-139 is helical when bound to heparin because all of the positively charged amino acids lie would on the same face of an a helix, thereby providing an array of cations to which the heparin may bind. In order to test this proposal, the peptide ATIII Random was prepared. ATIII Random has the same amino acid content as ATIII 123-139, but the sequence is rearranged so that the basic residues are evenly distributed around a helical cylinder.

Circular dichroism (CD) spectroscopy was employed to show that none of the peptide possessed secondary structure in aqueous solution. In trifluorethanol (TFE) and sodium dodecyl sulfate (SDS) ,the three peptides showed comparable degrees of a-helical structure.

In the presence of heparin, ATIII(123-139) and ATIII N135 GlcNAc were found to adopt a b-sheet like conformation, while the structure of ATIII Random was unaffected. In the presence of chondroitan-6 sulfate and dextran sulfate, two sulfated polysaccharides having negligible anticoagulent activity, b-sheet conformation was also induced in ATIII(123-139) and ATIII N 135 GlcNAc, but the structure of ATIII Random was again unaffected.

The complex was found to be saturable (1 to 20, peptide to heparin residues) and sensitive to NaCl. The peptide ATIII(123-139) was able to compete with the protein ATIII for binding to the active site on heparin as monitored by changes in the fluorescence enhancement of ATIII when bound to heparin. ATIII Random was less effective in inhibiting the ATIII-heparin interaction. A filter binding assay was employed to determine that the Kd's of the peptides for heparin were 3.5×10^{-7} M for ATIII(123-139) and ATIII N135 GlcNAc,and 5.4×10^{-7}M for ATIII Random. These values compare favorably to the heparin Kd of 1×10^{-7} M for human ATIII. The peptide ATIII(123-139) was shown to have the same affinity for crude heparin and heparin with high affinity for human ATIII.

Thesis Supervisor: Dr. Peter T. Lansbury, Jr.
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Unfortunately, I don't have the time to write that witty, yet sentimental acknowledgement section that I had hoped to include in this thesis. There are many people I would like to thank, I will try to do them justice in the short time I have.

First and foremost, many, many, thanks to my advisor Dr. Peter Lansbury. Five years ago when I was trying to decide which lab to join, many people (including my parents) encouraged me to work for Peter. Aside from the fact that his projects sounded very interesting and he was obviously going to be doing great science, I was told that being someones first student was a unique opportunity not to be passed up. So, I took the plunge and decided to join the group...or more accurately...become the group, along with Shimi Anisfeld, Kurt Halvorson, and Julia Hendrix.("Peter's Little People" as we were later dubbed at a conference). My conclusion 5 years later ? It was a great experience. Of course there were frustrating times and moments of doubt, but all of us got to see first-hand how ideas go from being a sketch on the blackboard to funded projects with publishable results. Furthermore, I was introduced to a research area, the structural biology of carbohydates, that I am really excited about pursuing in the future. Thank you Peter, for all your encouragement and understanding along the way.

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Saving the bestest for last...To Herr Doctore Luigi Raviolio...the left-handed genious (just like Leonardo daVinci)...known to the world as Laurent. No, you are the best discovery one could have made at MIT. You've made the last 3 years, the best ones yet. I'll love you forever. As soon as I finish off this page, we'll be off on our great adventure!

Here we go.................

Abbreviations Used in This Thesis

ATIII	Antithrombin III
Boc	t-Butoxycarbonyl
BOP	benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorphosphate
CD	circular dichroism spectroscopy
Cl_2-Bzl	2,6-dicholorobenzyl ether
Cl-Z	4-chlorobenzyloxycarbonyl
DCC	dicyclohexylcarbodiimide
DIC	diisopropylcarbodiime
DIEA	diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
FABMS	fast atom bombardment mass spectrometry
Fmoc	9-fluorneylmethoxycarbonyl
FTIR	Fourier Transform Infrared spectroscopy
GlcNAc	N-acetyl glucosamine
HOBt	hydroxybenztriazole
HOPip	N-hxydroxypiperidine
HPLC	high performance liquid chromatography
4-me-Bzl	4-methyl benzyl
MS	mass spectrometry
Mts	mesitylene sulfonyl
NMM	N-methyl morpholine
PDMS	plasma desorption mass spectrometry
SDS	sodium dodecyl sulfate
S-tBu	S-tert-butyl thio ether
S-S-tBu	S-S-tert butyl sulfonyl(mercapto)
TFA	trifuoracetic acid
TFE	trifluoroethanol
TFMSA	trifluormethane sulfonic acid

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Chapter 1.

Heparin and its Interaction With Antithrombin III

Heparin is a heterogeneous sulfated proteoglycan, the saccharide portion of which is widely used in the treatment of thrombosis.[1, 2] Approximately 33 metric tons (~500 million doses!) of heparin is processed for use worldwide each year. The anticoagulant activity of heparin is derived from its ability to activate several serine protease inhibitors which are critical in the regulation of the blood-coagulation cascade. [3] The primary physiological target of heparin is the inhibitor Antithrombin III (ATIII). Binding of the protein to a specific carbohydrate sequence in heparin induces a conformational change in ATIII and a 1000 fold increase in the rate at which ATIII neutralizes the proteases Factor X_a and thrombin. [4]

Despite its widespread use, the molecular details of how heparin binds and activate ATIII remain poorly understood. It is increasingly apparent that heparin has many biological functions in addition to its anti-coagulant activity. [5] A thorough understanding of the heparin-ATIII interaction is necessary for the design of synthetic anticoagulants which act in a more specific manner than the natural product. The interaction of ATIII and heparin is but one of many important proteoglycan-protein interactions. Because the functional aspects of the ATIII-heparin interaction have been studied extensively, the structural details of the ATIII-heparin binding event may serve as a model for other protein-proteoglycan interactions.

The primary objective of the project described in this thesis is to elucidate the molecular details of the heparin induced conformational change in ATIII. Uncovering the features that govern the heparin sequence specificity of the ATIII binding event is included in this objective. This chapter will first present background information about the structure and function of heparin. A discussion of the functional relationship between heparin and Antithrombin III will be followed by a survey of approaches used by others in understanding the conformational consequences of heparin binding to ATIII.

Heparin is a Distinctive Glycosaminoglycan

Proteoglycans are glycoproteins, the compostion of which is dominated by glycosaminoglycan carbohydrate chains. Glycosaminoglycan (GAG) chains are unbranched sulfated oligosaccharides composed of a repeating dissaccharide unit which includes an amino sugar, usually glucosamine or galactosamine. The GAG chains are linked to serine residues in the proteoglycan core protein via a neutral tetrasaccharide linkage sequence (GlcA-(β1-3)-Gal-(β1-3)-Gal-(β1-4)-Xylβ1-Ser). [6]

Proteoglycans are commonly classed according to the type of GAG chain they contain, although some proteoglycans have more than one type. Figure 1 illustrates some common glycosaminoglycan disaccharide units. Proteoglycan core proteins are divided into several classes based on their function. The proteins range in size from 20 to 200 kD [7]Although the amino acid sequences of relatively few proteoglycans are known, a S-G-X-G concensus sequence has been proposed as a xylosylation site for GAG chain attachment.[8]

The heparin disaccharide unit shown , α-L-idopyranosyluronic acid 2 sulfate (1-4) 2-deoxy-2-sulfamino a-D-glucopyranose 6-sulfate, is unique among the GAG chain types for a number of reasons. First, this disaccharide represents only the most common disaccharide unit found in the heparin GAG chain. Heparin is highly heterogeneous on the level of chain composition. A large number of important variations of the dissacharide are present in the heparin GAG chains in significant quantities. Glucuronic

acid is found in some disaccharides, rather than iduronic acid. The glucosamine residue may be N-acetylated or in the free amine form, rather than N-sulfated. In rare (but functionally important) cases, the 3-O position of the glucosamine may be sulfated in addition to the 6-O and N sulfation shown in figure 1.

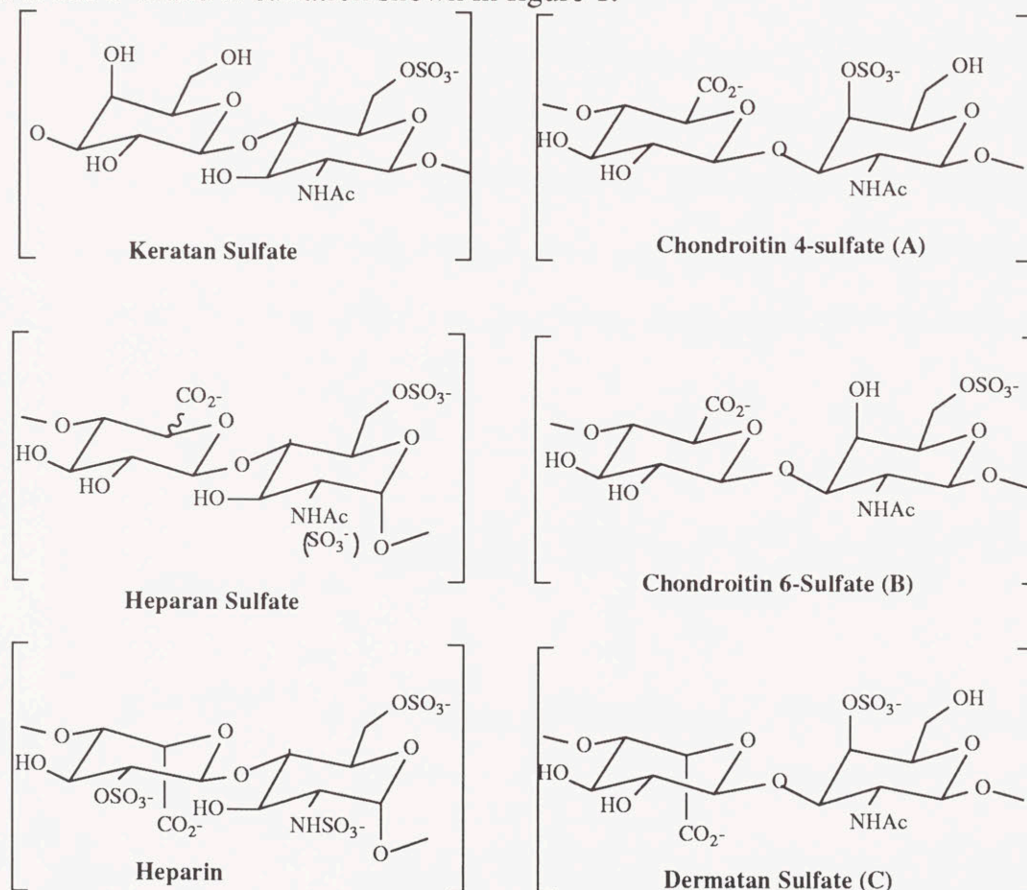

Figure 1. Glycosaminoglycan Dissacharide Units

A second distinctive feature of the heparin disaccharide is the fact that the uronic acid residue is found primarily in the iduronic epimer. While the N-acetyl glucosamine and glucuronic acid residues are found exclusively in the $^4C_1$ "chair " conformation, the $^4C_1$ and $^1C_4$ chair conformations iduronic acid are equi-energetic. ^{1}H-NMR studies of small heparin fragments (synthetic and derived from natural sources) have shown that iduronic acid residues (both sulfated and unsulfated) exist in a either a $^1C_4$ chair or a $^3S_0$ skew boat. [9, 10, 11]This finding implies that iduronic acid residues can act to maintain

flexibility in regions of the heparin chain, thus giving heparin its unique functional properties.

The biosynthesis of heparin is initiated by the assembly of the core protein and the O-glycosylation of selected serine residues with the neutral tetrasaccharide linkage sequence described previously.[12] The tetrasaccharide is elongated by the sequential 1-4 addition of α-D-N-acetyl glucosamine (GlcNAc) and β-D glucuronic acid (GlcA) residues. Each resulting carbohydrate chain is approx. 100 residues long. These chains are then sequentially N-deacetylated, N-sulfated, C-5 epimerized (glucuronic to iduronic acid), and O sulfated. The 3-O sulfation of glucosamine is believed to be the final step.[13] (see figure 2)

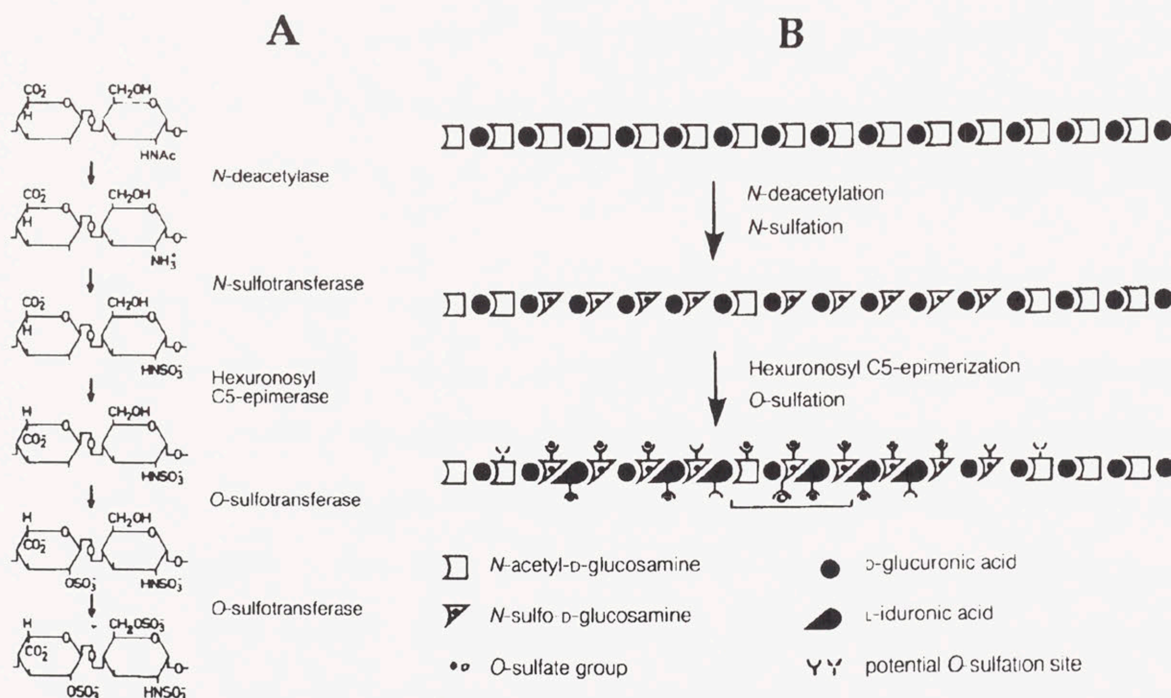

Figure 2

The heterogeneous composition of the heparin carbohydrate chains results from incomplete processing of the chain with the modifications described above. If each of the steps were complete, the only disaccharide observed would be the structure shown in figure 1 plus an additional 3-O sulfate residue. To date the enzymes involved in the

elongation and modification of the chain have been identified, but not recovered (from either natural or genetically engineered sources) in large enough quantities to permit study their specific functions. Analysis of the distribution of the possible variations reveals that the post polymerization modifications are randomly distributed through out the heparin GAG chains.[14]

It is worth noting that the related GAG chain, heparan sulfate, contains all of the same saccharide modifications as heparin. Heparan sulfate has been proposed to be an unprocessed form of heparin. [15] Generally, heparan sulfate has more GlcNAc residues and glucuronic acid residues than does heparin. Heparan sulfate also has fewer sulfated residues. Many different definitions have been cited in attempts to define the differences between two types of GAG chains.[16, 17] Kjellan and Lindahl have suggested that the term heparin be reserved for those GAG chains biosynthesized in mast cells, and the term heparan sulfate be applied to heparin-like GAG chains derived from all other proteoglycans.[7]

Little information is available regarding the macromolcular solution conformation of the heparin GAG chains. Computer modeling of X-ray diffraction data from oriented heparin fibers shows an extended helix with an axial rise of 0.84 nm per disaccharide.[18] (See figure 3). ^{1}H-NMR studies of heparin fragments have shown that there can be substantial sequeence dependant variations in the iduronic acid residue ring conformations along the chain. [9] These variations in ring conformation alter the length of the dissachride repeat in the helix. However, the lack of measurable long range interactions in the heparin polysaccharide chain make it is impossible to deduce the macromolecular chain conformation using NMR spectroscopy.

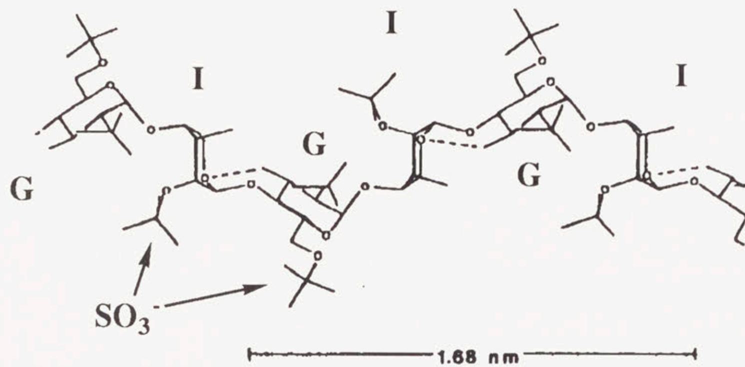

Figure 3. Proposed Conformation of the Heparin GAG chain
G, 2-deoxy-2-sulfamine glucose 6 -O-sulfate I, Iduronic acid 2-O-sulfate

In general, proteoglycans are found in intracellular granules, associated with cell membranes (either as transmembrane proteins or via phosphoinositol linkers), or in the extracellular matrix. [5, 19] Their functions are highly diversified, yet poorly understood roles in cell-cell interactions, cell motility, and morphogenisis. Several comprehensive reviews of proteoglycan function have been published recently.[5, 7, 18] Recently attention has be devoted to the finding that cell surface proteoglycans possessing heparan sulfate GAG chains mediate the binding of various growth factors, e.g. endothelial derived growth factor (EDGF), to their respective growth factor receptors. [20]

Heparin proteoglycans are found primarily in mast cell granules.[7] Mast cells are found in connective tissues and in the walls of blood vessels. The heparin proteoglycan protein core is a member of the serglycin family of core proteins. These proteins (17-19 kD) contain up to 24 ser-gly repeats in which each serine is substituted with a GAG chain. Shortly after assembly, the proteoglycan is degraded by specific peptidases and β-glucuronidases resulting in the release of an ensemble of heparin GAG chains ranging in MW from 5,000 to 25,000. A number of different functions have been proposed for endogenous granule heparin including packing histamine, binding and inhibition of granule proteases, and regulating complement activation. On this level too, heparin is unique from other GAGs in that it functions after release from the core protein. Upon

degranulation, heparin GAG chains are released from the mast cells. It is unclear whether this released endogenous GAG-heparin plays any role in regulating blood flow.

Through out the rest of this thesis, the heparin GAG chains will be refered to as "heparin".

Exogenous Heparin Activates Protease Inhibitors in The BloodCoagulation Cascade

In 1916, Jay McLean , a medical student at Johns Hopkins looking for substances that cause blood to clot, isolated a factor from mammalian tissue which prevented blood coagulation.[1] McClean's research supervisor immediately recognized the potential of such a substance in the treatment of coagulation disorders. By the 1920's several groups were manufacturing and marketing heparin. Today, as then, heparin is isolated from mammalian tissues high in mast cells, such as porcine intestinal mucosa and bovine lungs. Despite its almost immediate acceptance as a therapeutic agent, it took until 1968 before the primary constituents of heparin were identified as N-sulfated glucosamine and L-iduronic acid.[1]

Heparin acts as an anticoagulent by activating several protease inhibitors in the blood coagulation cascade. The primary physiological target of heparin is believed to be the serine protease inhibitor Antithrombin III.[3, 21] ATIII can inhibit a number of serine proteases in the blood clotting cascade, however thrombin and factor Xa are believed to be ATIIIs primary physiological targets. [4] (See figure 4) Factor Xa cleaves prothrombin generating thrombin. Thrombin acts in the penultimate step in clot formation, cleaving fibrinogen into fibrin. Fibrin subsequently polymerizes into a gel like substance which is the basic material of a clot.

Heparin accelerates the rate at which ATIII binds to and neutralizes its target proteases. Jordan et al [4]In the absence of heparin, ATIII forms a slowly reversible inhibitory complex with thrombin at a second order rate constant of $4.25 \times 10^5 M^{-1} min^{-1}$. For complexation with factor Xa this rate is $1.88 \times 10^5 M^{-1} min^{-1}$ in the absence of heparin. The inhibitory complexes are very stable, having half-lives on the order of

days. In the presence of heparin, the rates of inhibitory complex formation are accelerated to 1.7 x $10^9 M^{-1} min^{-1}$ and 2.4 x $10^8 M^{-1} min^{-1}$ for thrombin and factor Xa respectively. The heparin induced rate enhancements correspond to a 4000 fold increase for thrombin and 1,200 increase for factor Xa.

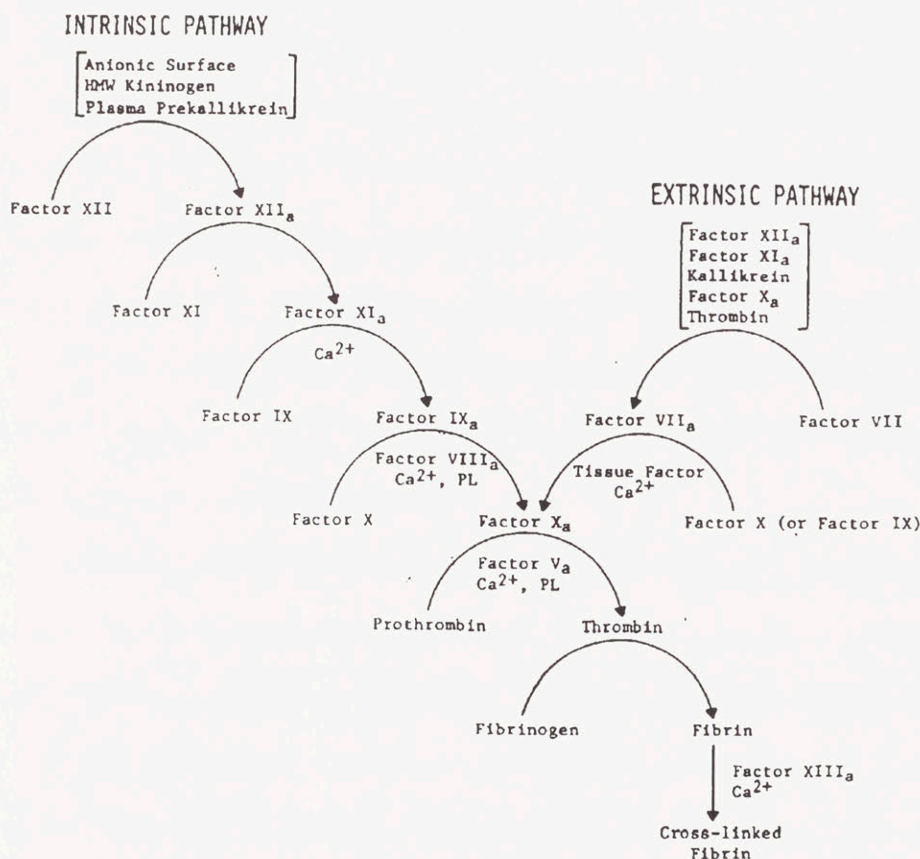

Figure 4. Blood Coagulation Pathway

Heparin accelerates the rates of ATIII-factor Xa and ATIII-thrombin complexation by two slightly different mechanisms In ATIII-factor Xa complex formation, heparin need only bind ATIII. [22] Heparin fragments as small as a pentassacharide will accelerate the ATIII-factor Xa complexation *in vitro*. [23] The conformational change induced in ATIII by heparin increases the affinity of ATIII for factor Xa In ATIII-thrombin complex formation, heparin acts as a template to which both ATIII and thrombin bind. [24, 25] The rate of complexation is accelerated in part by limiting the diffusion of ATIII and thrombin. Only heparin fragments 18 saccharides or

longer can accelerate complex formation. [26] The fact that heparin forms a ternary complex with ATIII and thrombin, is not incompatible with the idea that the heparin induced conformational change in ATIII aids the interaction of ATIII and thrombin.

Heparin is currently administered as an antithrombotic/anticoagulant either intraveinously or subcutaneously. Both practices require hospitalization. Heparin is also used with many extracorporeal devices such as kidney dialysis or heart -lung machines. In these cases, large quantities of heparin must be added to the patients blood to prevent coagluation upon contact with the foreign surfaces. Eventually, the heparin must be neutralized, a task usually accomplished with protamine, another drug with negative side effects. Much research is in progress to develop bio-compatible polymers with covalently-linked heparin. Such heparin would obviate the need for both heparin and protamine when using extrcorporeal devices.

Since its original application as an anticoagulant, many other biological properties have been attributed to exogenous heparin. The list includes inhibition of angiogenisis, antilipemic behavior, sequestering of growth factors in the extracellular matrix, and modulation of gene expression. However, many functions that have been proposed for heparin are based on data indicating that heparin binds various proteins. Given that sulfated heparin is one of the strongest known natural acids, it is expected that any protein with a high concentration of positive charges will have affinity for heparin. Mere affinity for heparin is not definitive proof of a functional relationship. The increasing number of non-anticoagulent functions attributed to heparin only accentuates the need to clearly define the ATIII-heparin interaction so that more specific agents can be developed.

Antithrombin III Binds a Specific Pentasaccharide Sequence Within the Heparin Chain

In 1976 Lam *et al.* reported that ATIII binds to only 1/3 of the heparin GAG chains isolated from a given heparin preparation. "Crude" heparin could be separated into aliquots having either high affinity or low affinity for ATIII. In experiments using

heparin octasaccharides, the dissociation constant (K_D) of ATIII for high affinity heparin was determined as 1×10^{-8} M Low affinity heparin octasaccharides, which also lacked biological activity, had a K_D of 5×10^{-4} Mheparin. [27]

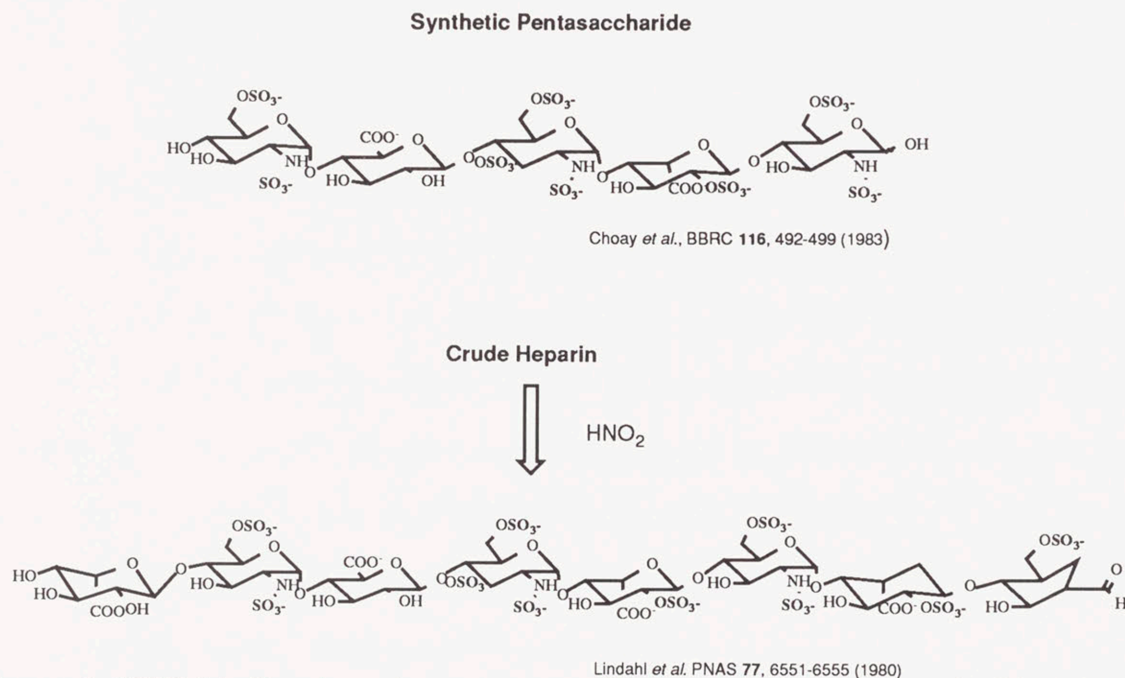

Figure 5 ATIII binding heparin sequences. Top: synthetic pentasaccharide Bottom: shortest oligosaccharide sequence that can be isolated from natural sources and still contain the pentasaccharide.

In the following years, as the result of extensive analyisis of chemically and enzymatically derived heparin fragments having high affinity for ATIII, it was determined that ATIII specifically recognizes a pentasaccharide sequence within the heterogeneous heparin chains.[13, 28, 29, 30, 31, 32] (See figure 4). This pentasaccharide sequence contains two rare residues; a glucosamine residue sulfated at the 3-O, 6-O and N positions and an unsulfated glucuronic acid. The work directed towards establishing the identity of this sequence culminated in the total synthesis of the pentasaccharide reported by Jean Choay *et al.* in 1983.[33]

In order to fully appreciate the work done in proving the identity of the ATIII binding site on heparin, one must understand the technical challenges of working with the sulfated proteoglycans. Compared to manipulating peptides or oligonucleotides, it is

extremely difficult to isolate and identify heparin fragments from natural sources. The primary obstacle in the structural analysis of heparin are the chemically labile sulfate groups. Ironically, it is apparent that the location of sulfate groups confers specific biological activity on a given polysaccharide sequence.[13, 23, 30] The heparin polysaccharide is cleaved into fragments using either nitrous acid or a bacterial heparinase.[34, 35] Both methods generate fragments in multiples of the disaccharide unit. Only cleavage by nitrous acid will leave the ATIII-binding site intact.[35] Using either HPLC based or gel electrophoretic techniques, small (less then 8 saccharide) heparin fragments can be isolated. The chromatographic properties of the possible heparin sequences are not sufficiently different to allow the isolation and purification of heparin fragments greater than 8 saccharides by existing techniques. Once purified, the fragment can be characterized using a combination of ^{1}H and ^{13}C NMR [36, 37] and elemental analysis of both the fragment and its disaccharide components. The sulfate groups prohibit the use of most mass spectroscopic techniques.

The synthesis of a heparin fragments is lengthy and difficult because of the site specific sulfation requirements as well as the presence of both α and β -glycosidic linkages. The first reported synthesis in 1983 of the ATIII-binding pentasaccharide involved 75 steps.[33] Several shorter syntheses (40 step) have since been reported. [38]

Heparin Binding Induces a Conformational Change in Antithrombin III.

In early investigations of the physico-chemical properties of ATIII, it was discovered that the circular dichroism spectrum of ATIII complexed to heparin was distinctly different from that of ATIII alone, indicating that heparin alters the conformation of ATIII upon binding. Several different methods have been used in attempts to define the nature of the heparin induced conformational change.

In following the chemical denaturation of ATIII in the presence and absence of heparin using CD spectroscopy, Villanueva *et al.* found that secondary structure of high α-helical content was stabilized by heparin. [39, 40] The intrinsic fluorescence of ATIII was

also found to increase 30% upon heparin binding. This intrinsic fluorescence enhancement was later shown conclusively to result from changes in the environment of tryptophan residues buried in the protein. [41] A number of NMR studies of ATIII in the presence of native heparin as well as synthetic heparin fragments have been reported. [41, 42, 43, 44] These studies reveal pertubations of surface histidine residues as well as many presumably buried aromatic residues upon heparin binding. However, the size of the protein (58,000 D) precludes sequence assignments of those resonances perturbed by heparin binding.

The most influential ideas concerning the nature of the conformational change in ATIII have come from studies of the crystal structure of a related protease inhibitor, α1-anti-proteinase, which will be duscussed in the next section.

Alpha 1-antiproteinase as a Model for ATIII?

ATIII is a member of the "serpin" class of serine protease inhibitors. Serpins inhibit serine proteases via formation of a stable complex with the protease. Ultimately the serpin is cleaved and the complex dissociated. [45] Unlike other protease inhibitors, the cleaved serpin has no affinity for the native proteases and the cleavage reaction cannot be run in reverse to re-ligate the protease cleavage site. The serpin class of inhibitors includes the α1-antiproteinase inhibitor, α1-antichymotrypsin inhibitor and the C1 inhibitor. [46] Ovalbumin has striking sequence homology to the serpins, but has no known protease inhibitory activity.

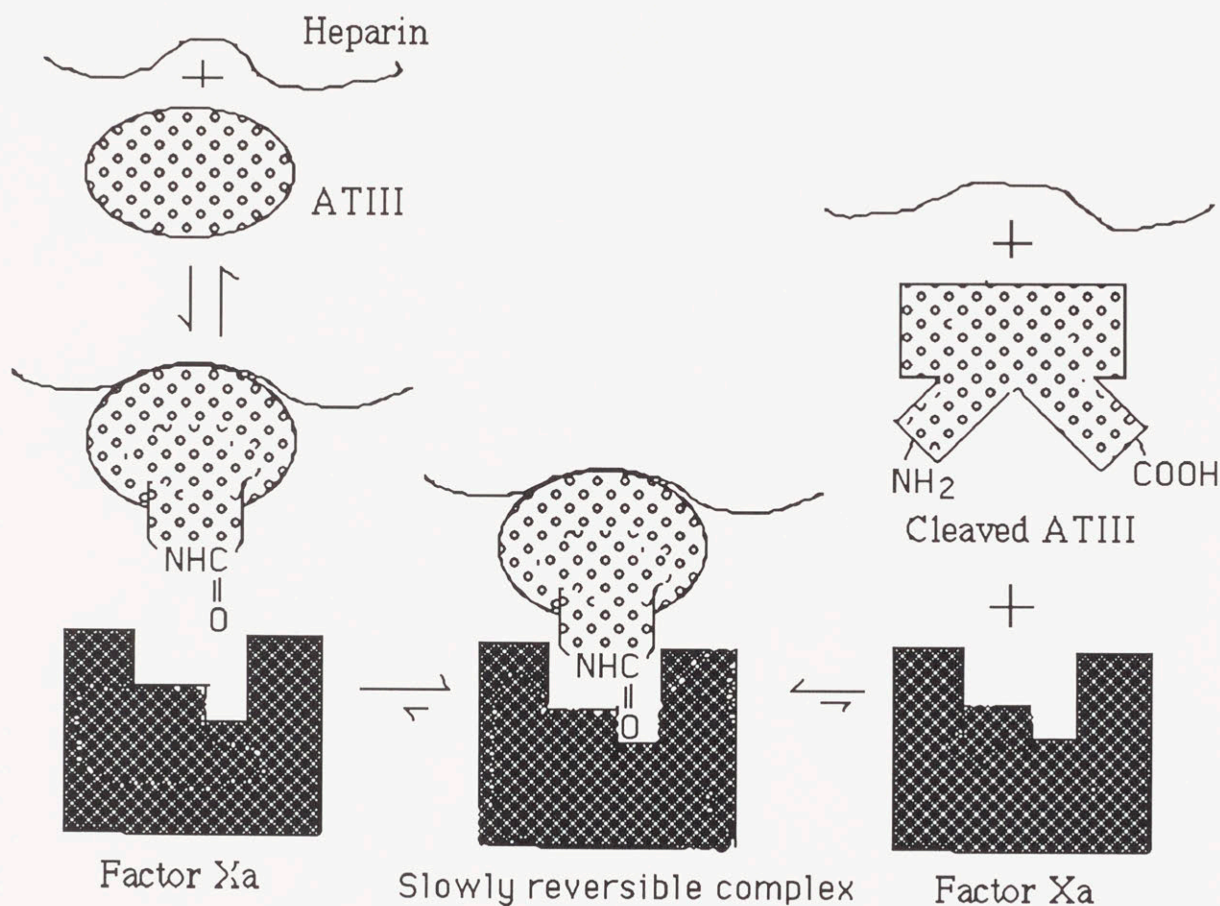

Figure 6 Schematic diagram of the interactions between ATIII, heparin, and a target protease, Factor X_a.

Before being released from the protease complex, ATIII is cleaved by thrombin or Factor Xa at the same site at which it bound the protease.(See figure 6) Like other serpins, the cleaved form of ATIII has different hydrodynamic properties and is more stabile to heat denaturation than the native form of the protein. Cleaved ATIII has a 500 fold less affinity for heparin than does the native ATIII. [47] These findings suggest that the cleaved form of ATIII has a different conformation than the native form

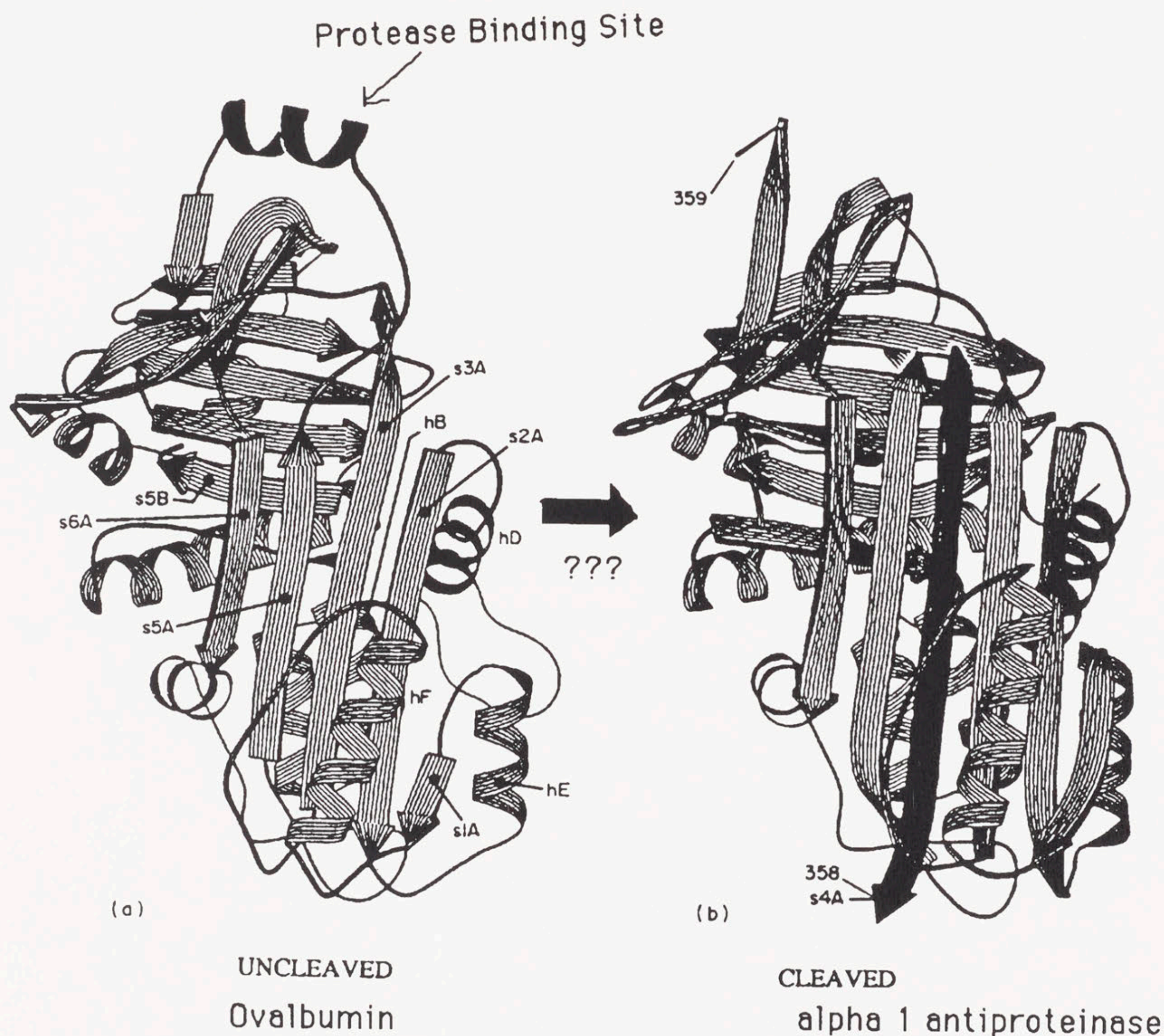

Figure 7. a) Ovalbumin b) cleaved α1-antiproteinase inhibitor

The only crystallographic data available for any of the serpins in any form,cleaved or uncleaved, is of the cleaved α1-antiproteinase inhibitor. The 3 Å resolution crystal structure of this inhibitor was reported in 1984.[48] (See figure 7) This crystal structure reveals that the amino acids at either side of the protease cleavage site (358 and 359) are 70 Å apart. A crystal structure of the homologous protein ovalbumin was reported in 1990. [49] Despite its lack of protease inhibitory function, ovalbumin has been cited as a model for the uncleaved form of the α1-antiproteinase inhibitor and other serpins. [50] A

comparison of the two crystal structures suggests that once the protease inhibitory loop is cleaved, the released strand could inserts itself into the middle of the β-sheet structure as shown in Figure 7b.

The amino acid sequence of ATIII projected on to the crystal structure of the cleaved α1-anti-proteinase inhibitor was reported by Huber and Carrell as a model for the cleaved form of ATIII. [51] The region in the ATIII sequence encompassing residues 120-150 has been implicated in heparin binding by a number of biochemical labeling studies of the protein [52, 53, 54](to be discussed more thoroughly in Chapter 2 of this thesis). When projected on the crystal structure of the α1-antiproteinase crystal structure, these residues are found on an α-helix. Huber and Carrell proposed that this helical region is the heparin binding site. (See Figure 8)

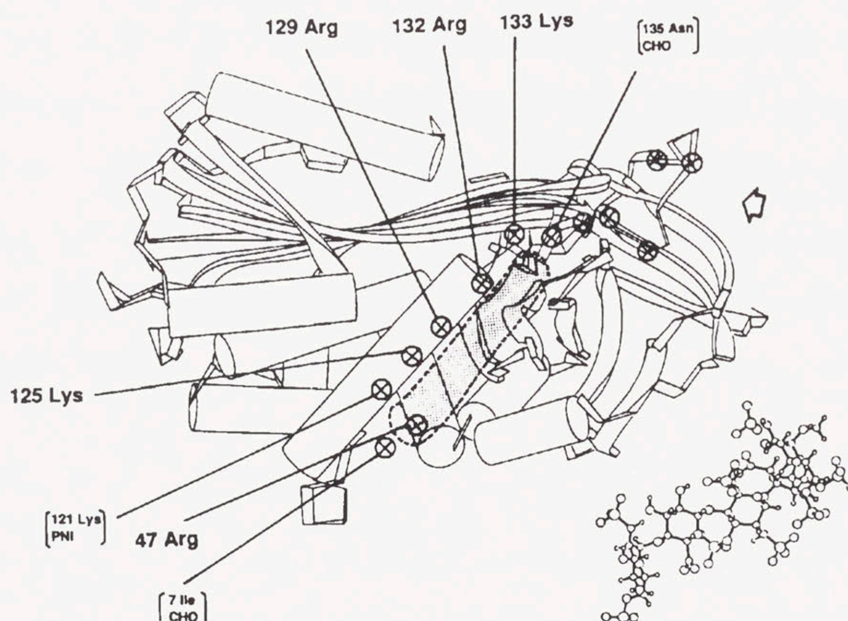

Figure 8. The sequence of ATIII projected on the structure of α1-antiproteinase. The β-sheets shown on the front of the strucutre in figure 5b are on the top and seen from the side. The residues implicated in heparin binding are highlighted. The carbohydrate structure on the right is a model of the ATIII binding heparin pentasaccharide.

This model ATIII structure derived from α1-antiproteinase is consistent with the Villanueva's proposal that α-helical structure is involved in the heparin in the binding site. Moreover, van Boeckel and Grootenhuis have used this ATIII model structure in molecular modelling experiments to dock a minimized structure of the heparin

pentasaccharide sequence to the proteins surface thereby "defining" the contacts between the amino acid side chains and particular sulfates with the pentasaccharide.[55]

This model protein structure has been cited extensively in the literature regarding the ATIII-heparin binding event. However, the model has several short-comings that should not be overlooked. This model is based on a non-functional protein Neither α1-antiproteinase (either native or cleaved) nor the cleaved form of ATIII has significant affinity for heparin. [47, 55] Even if the cleaved α1-antiproteinase structure does approximate the structure of cleaved ATIII, it seems improbable that the structure could accurately describe a binding function that α1-antiproteinase doesn't have. Furthermore, the model does not account for any of the conformational change that is reportedly induced in the protein upon heparin binding.

A crystal structure is a relatively rigid model, whereas the spectroscopic studies of heparin binding to ATIII suggest that ATIII is a conformationally mobile protein. We therefore chose to model the interaction using synthetic peptides, as will be described in the following chapter. Monitoring the behavior of a synthetic peptide in the presence and absence of heparin, may allow us to observe those conformational changes induced in ATIII by heparin binding.

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Chapter 2.
A Peptide Model

Introduction

Chapter 1 described several studies of the heparin induced conformational change in Antithrombin III; each of these studies has its limitations. While the spectroscopic studies monitor global properties of the heparin-Antithrombin III complex, they provide little specific information about which residues are involved in the interaction.[1, 2, 3] The ATIII model structure based on α1-antiproteinase identifies a potential structure for the heparin binding site but can not account for the conformational change. [4] We chose to model the complex using synthetic peptides. Our goal was to create a peptide-heparin complex which would mimic the conformational changes of the ATIII-heparin interaction. The behavior of this complex could provide clues as to how heparin alters the conformation of ATIII as well as how ATIII recognizes specific oligosaccharide sequences of heparin.

In this chapter, some of the issues surrounding the use of peptides in studying protein-ligand interactions will be discussed. Additionally, the peptide sequences chosen to model the heparin binding site on Antithrombin III will be presented.

Common Approaches to the Study of Protein-Ligand Interactions.

In order to fully understand the conformational consequences of any protein-ligand interaction, one would ideally like to know the conformation of the protein in both its free and ligand-bound states. For example, high resolution crystal structures have been solved for a number of antibodies both in the presence and absence of their antigens (e.g. antibody-lysozyme[5] and antibody-neuramidase[6] complexes). These antibody-antigen complex crystal structures reveal details about the atomic contacts comprising the binding site. Comparing the structure of the antibody alone to that of the complex allows one to define the conformational changes induced in the antibody (either backbone or side chains movements) that may occur upon antigen binding. Unfortunately, many protein-ligand interactions are not amenable to such complete investigations, simply because the protein or the complex cannot be crystallized. In theory, ^{1}H-NMR spectroscopic studies of a protein and its ligand complex could provide similar proof of the structural consequences of ligand binding.

In the absence of a protein-ligand cocrystal, many researchers have tried to infer the conformational properties of a protein-ligand interaction from the crystal structure of the protein alone. Genetic engineering is commonly used in conjunction with crystallography in the study of protein-ligand interactions. For example, if a protein has been cloned and expressed, mutations in the amino acid sequence thought to be important for the protein-ligand interaction can be made. The functional properties (binding, ligand selectivity, turn-over rates, etc.) of the mutant proteins are determined. The changes in function can be correlated with changes in the sequence of the native protein. These mutant proteins may also be crystallized and their structures compared to that of the native protein. The mutations may be instrumental in elucidating regions of the protein and or amino acid residues critical to ligand binding. However, none of the findings will constitute definitive proof of the conformation of the ligand bound protein.

The Antithrombin III-heparin interaction is a particularly challenging protein-ligand interaction to study. To date, no crystal structure of Antithrombin III in any form

has been reported.[4] The crystal structure of the cleaved α1-antiproteinase inhibitor has been cited as a model for the structure of ATIII. However, as described in the previous chapter, α1-antiproteinase does not bind heparin (nor does ATIII when cleaved).[4] Therefore, the model must be interpreted with caution. In addition, although human antithrombin III has been cloned and expressed in both mammalian and yeast cells, its expression levels are too low to allow the study of genetically engineered protein.[7, 8]

Because heparin has been shown to alter the conformation of Antithrombin III upon binding[1, 3], it is critical to design experiments in which one can directly observe the heparin binding event. We, therefore, chose to model the protein-ligand interaction using synthetic peptides derived from a putative heparin binding site on Antithrombin III. The conformation of these peptides can be examined using several different spectroscopic techniques both in the absence and in the presence of heparin.

Synthetic Peptides as Tool for Studying Protein-Ligand Interactions

With the widespread availability of automated peptide synthesizers, research groups from many biomedical disciplines have begun to routinely use synthetic peptides in the study of protein function, protein folding, and protein-ligand interactions. The use of a synthetic peptide to model the conformational behavior of a protein raises a number of questions about the solution conformation of peptides and ultimately about the forces contributing to the tertiary structure of a protein. For example, given that small peptides (e.g. <30 amino acids) often have no stable secondary stucture in aqueous solution, what assumptions can be made when relating the structural or functional behavior of the peptide model to that of the native protein?

Many different forces contribute to the stable secondary and tertiary conformation of a globular protein. These forces include electrostatic interactions, hydrogen-bonding, and hydrophobic interactions.[9] These forces can be viewed as ranging from "local" interactions between neighboring residues in the polypeptide chain, to "long range"

interactions between different regions within a polypeptide chain which only come together once tertiary structure has formed.[10]

Short peptides (< 20-30 amino acids), whose sequences are derived from globular proteins, rarely assume stable secondary structure in aqueous solution. Notable exceptions to this generalization are the "S" and "C" peptides derived from ribonuclease A.[11, 12] The energetic and entropic barriers to formation of ordered structures like those found in a fully folded protein (α-helices, β-turns and sheets) are too great to be overcome by most peptides in aqueous solution. For example, in order to form an α-helix, the first three amino acids in the chain must give up their rotational freedom to twist into the helical conformation. Because these first three residues are not involved in the internal hydrogen bonding that stabilizes the rest of the helix, initiation of this structure is unfavorable.[13]

There is both structural and functional evidence supporting the idea that short peptides may exist in transient conformations or have conformational preferences. Physical evidence for the presence of order in small peptides was described by Scheraga in NMR studies of tetrapeptides derived from α-chymotrypsin.[14] The sequences described have measurable β-turn structures in solution similar to the structure of the analogous sequence in the native protein. In recent years, multi-dimensional NMR experiments performed by Wright and Dyson have also shown that transient α-helical and β-turn structures exist in a number of peptides in aqueous solutions.[15]

Functional evidence for short peptide conformations was derived from the use of synthetic peptides in stimulating antibody production. It was initially believed that synthetic peptides were sampling a large number of conformations in solution on a nanosecond timescale.[16] Statistically, one of these conformations would induce production of an antibody which recognizes the protein from which the peptide sequence was derived. In 1983, Niman *et al.* showed that the proportion of antibodies produced by a given peptide that recognizes both the peptide and the protein from which the sequence

was derived, was too large (approx. 25 %) to be explained by the stochastic model.[17] It was concluded that either the peptides have greater conformational preferences in solution than previously supposed or that the antigenic regions of proteins are more flexible. There is evidence that both conclusions may be correct. The NMR studies discussed previously describe conformational preferences of short peptide sequences. Analysis of temperature factors in high resolution crystal structures has revealed regions of flexibility on some protein surfaces.[18]

Ligand binding has been shown to stabilize structure in small peptides. In theory, the peptide conformation bound by the ligand need not be a prevalent peptide conformation in solution. The Curtin-Hammett postulate states that if the rate of ligand binding is much slower than the rate of conformational interconversion, a peptide conformation that exists only a minor fraction of the time in solution may be the major conformation in the ligand bound state.[19] Assuming that some population of the peptide exists in a conformation resembling the protein and that the ligand binds in a conformationally specific manor, a peptide may bind a ligand with a conformation resembling that of the native ligand bound protein.

Synthetic peptides have been used in the study of many protein-ligand interactions. For example, peptides derived from the mitochondrial protein signal sequences have been shown to form amphipathic α-helices in the presence of phospholipids as do the native proteins.[20] Because the interaction of ATIII and heparin results in a conformational change in the protein, it is critical to employ a method that will mimic the flexibility of the native system. In theory, a peptide derived from the putative heparin binding site on ATIII may mimic the conformation of ATIII when bound to heparin. In addition, the small size of this peptide can aid the identification of those features important for the interaction.

A consequence of this reductionist approach is that the peptides, being far more flexible that the native protein, may bind the ligand in some non-native form. The

peptide model also cannot mimic tertiary structure that may be involved in binding. Since the conformation of heparin bound ATIII has not been determined by an independent method, we have no way to definitively prove the relevance of any structure in the model peptides.

Residues 123-139 from human Antithrombin III were chosen as the sequence to study.

At the outset of this research project, the first task was to determine which sequence of the protein to synthesize. Three regions in the ATIII sequence have been proposed to be associated with heparin binding. A common feature of each of these regions is the high density of positively charged residues.

ATIII Sequence	
(123-139)	...FAKLNCRLYRKANKSSK...
(287-298)	...KPEKSLAKVEKELT...
(39-59)	...KIPEATNRRVWELSKANSRFA...

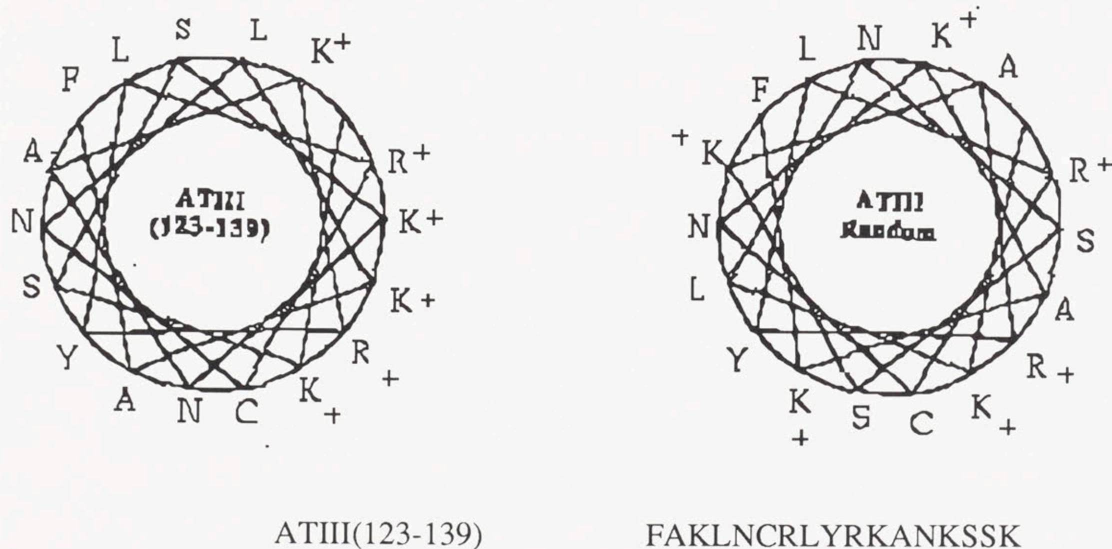
Figure 1. Putative heparin binding sites. The underlined residues indicated known points of mutation which effect ATIII activity.

One such region includes amino acids 105-150. Four lysine residues in this region, K107, K114, K125, and K136, are protected from chemical modification by heparin binding.[21, 22, 23] Arginine labelling experiments show that Arg 129 and Arg 145 may be involved in binding as well. [24] Proteolytic fragments of ATIII containing this region of the sequence (114-156) have been shown to have a high affinity for heparin.[25] In addition, polyclonal antibodies raised against a synthetic peptide based on the sequence 124-145 have been show to block heparin binding and to partially activate ATIII for thrombin inhibition.[26] Reduction of the Cys 128-Cys 8 disulfide bond prevents activation of ATIII.[27] Finally, heparin binds more strongly to an ATIII variant lacking N-glycosylation at Asn 135 than to the native glycoprotein.[28]

The ATIII sequence surrounding Arg 47 may comprise an alternative or second heparin binding region, as it also has a high concentration of positively-charged residues. Naturally-occuring ATIII variants having mutations at R47 (R47H, R47C) and P41

(P41L) have been isolated from patients with clotting disorders and heparin binding has been shown to be effected.[29, 30, 31, 32] A third region on the ATIII sequence comprising residues 281-292 has also been cited as a possible heparin binding region. [33]

In a report by Villanueva *et al.* in 1984, CD spectroscopy was used to monitor the chemical denaturation of ATIII in the presence and absence of heparin.[33] These studies suggested that heparin binding stabilized α-helical structure. The sequence of 281-292 was cited as a possible heparin binding site because if this sequence were displayed on a helical wheel diagram, all the positively charged residues would be segregated on one face of the helix. It was proposed that such a sequence may not be helical (or not in a stable helix) in the native protein structure because of the potentially unfavorable side chain electrostatic interactions. In the presence of a neutralizing polymer such as heparin, helical structure would be stabilized. Poly-L-Lysine and poly-L-Arginine were found to assume helical structure (as determined by CD spectroscopy) in the presence of heparin or chondroitin-sulfate.[34, 35] This finding supports the idea that heparin can induce structure in a charged peptide, but does not address the question of the specificity of the heparin-ATIII interaction. Upon inspection of the sequence ATIII 123-139, one finds that the cationic residues in this sequence would also be segregated on one face of a helix if this sequence were helical. See figure 2.



ATIII(123-139) FAKLNCRLYRKANKSSK

ATIII Random FKAKNCRLYRAKSSNLK
ATIII N135 GlcNAc FAKLNCRLYRKAN*KSSK
* N-acetyl glucosamine

Figure 2.

The convergence of the experimental evidence implicating the sequence 105-150 in binding with the finding that the cationic residues in sequence 123-139 segregate to one face of a helical wheel, led to the focus of our peptide model on the ATIII 123-139 sequence. Ultimately, three synthetic peptides whose amino acid composition was derived from ATIII 123-139 would be studied.

The first peptide to be prepared represented the native sequence. Structural and binding properties of this peptide were to be the model for the protein-heparin interaction. Our hope was that this peptide would bind to and adopt helical structure in the presence of heparin as proposed by Villanueva. In order to test the idea that the positioning of the basic residues within the sequence was essential for the formation of helical structure, a second sequence was planned in which the residues were rearranged. This sequence permutation, named ATIII Random, was planned such that the basic residues would no longer segregate to the same face of a helix.(See figure 2). It was later realized that "ATIII Random" is somewhat of a misnomer because there is nothing truly "random" about the changes in the sequence of this peptide. The term "random" is in reference only to the distribution of the positively charged residues around the helical wheel. The third peptide prepared and studied, ATIII N135 GlcNAc, had the same sequence as ATIII(123-139) but Asn 135 was glycosylated with a single N-acetyl glucosamine residue in a very preliminary attempt at studying the effects of glycosylation of ATIII on the proteoglycan-protein interaction.

An additional peptide sequence, named ATIII 2E, was also planned in which 2 cationic residues would be replaced with an anionic residue (Glu). The rationale behind this change is that the previously unfavorable clustering of residues would be changed to a series of potentially stabilizing salt bridges. Although the synthesis of this peptide is

mentioned in the Chapter 3, this peptide was not fully purified and no structural studies were performed with it.

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Chapter 3

Peptide Synthesis

The peptide sequences presented in chapter 2 were synthesized manually using solid phase peptide synthesis techniques. In theory, the assembly of a polypeptide on a solid support is accomplished in the following manner. An amino acid with a reversible α-amino protecting group (BOC or FMOC) and suitable side chain protection is linked to an insoluble resin through the amino acid α-carboxyl group. The peptide chain is grown in the C to N direction by the sequential removal of the α-amino protecting group at the end of the resin bound peptide and the addition of an α-amino protected amino acid (or peptide fragment) with the appropriate coupling reagents. Ultimately, the peptide is cleaved from the resin and all protecting groups removed.

Despite the simplicity of the above description, peptide synthesis is only successful when the protecting groups, resin linkages and coupling chemistry function as intended. In addition, solvation of the resin bound peptide product can be problematic while purifying and positively identifying the product is a far from trivial matter. [1]

The peptides planned from Antithrombin III were not expected to be difficult sequences to prepare. A peptide seventeen amino acids long was well within the limits of peptide syntheses commonly reported at the time this project was initiated. The

sequences contained few hydrophobic residues which might otherwise cause solubility problems.[2]

The goal for this project was to prepare the peptides in sufficient quantities to perform a number of different structural studies and binding experiments. Therefore, little effort was made to optimize the chemistry used. However, a high standard was set for the purification and full characterization of the peptide products. This was to ensure that at no time would the results of any experiment be questioned because of the chemical integrity of the peptides synthesized.

This chapter will cover the strategies and techniques used in preparing the peptide sequences. Some of the difficulties encountered in the syntheses will also be presented and discussed.

<u>Different Strategies Were Undertaken in Synthesizing the Peptides.</u>

Two different synthetic strategies were employed in the preparation of the peptides, the stepwise method and the fragment condensation method. A schematic diagram of the two strategies is shown in figure 1. In the stepwise method, each amino acid residue is coupled sequentially to the resin until the peptide chain reaches the desired length. The peptide is removed from the resin, deprotected and purified. In the fragment condensation method, shorter peptide sequences are assembled on the resin. These short sequences or fragments, are removed from the resin in a manner leaving the side chain protecting groups in place and purified. The purified fragments are then coupled together on the resin to generate the desired peptide. The final peptide is cleaved from the resin and fully deprotected.

An inherent problem in stepwise peptide synthesis is that although the efficiency of the individual coupling reactions may be greater than 99%, the theoretical yield of the desired product decreases as a function of the number of sequential couplings involved.[1] For example, a 40 amino acid peptide prepared sequentially with 99% yield at every step, would have maximum theoretical yield of the completed peptide of 67%.(0.99^{39})

In addition there are 39 different potential single residue deletion impurities from which the desired product must be isolated (not mention impurities from incomplete deprotection etc.)[1, 3].. There is also evidence suggesting that at longer chain lengths (or extremely hydrophobic sequences), resin bound peptide will aggregate, thereby inhibiting both the elongation of the chain and quantitation of the coupling reactions.[4, 5, 6]

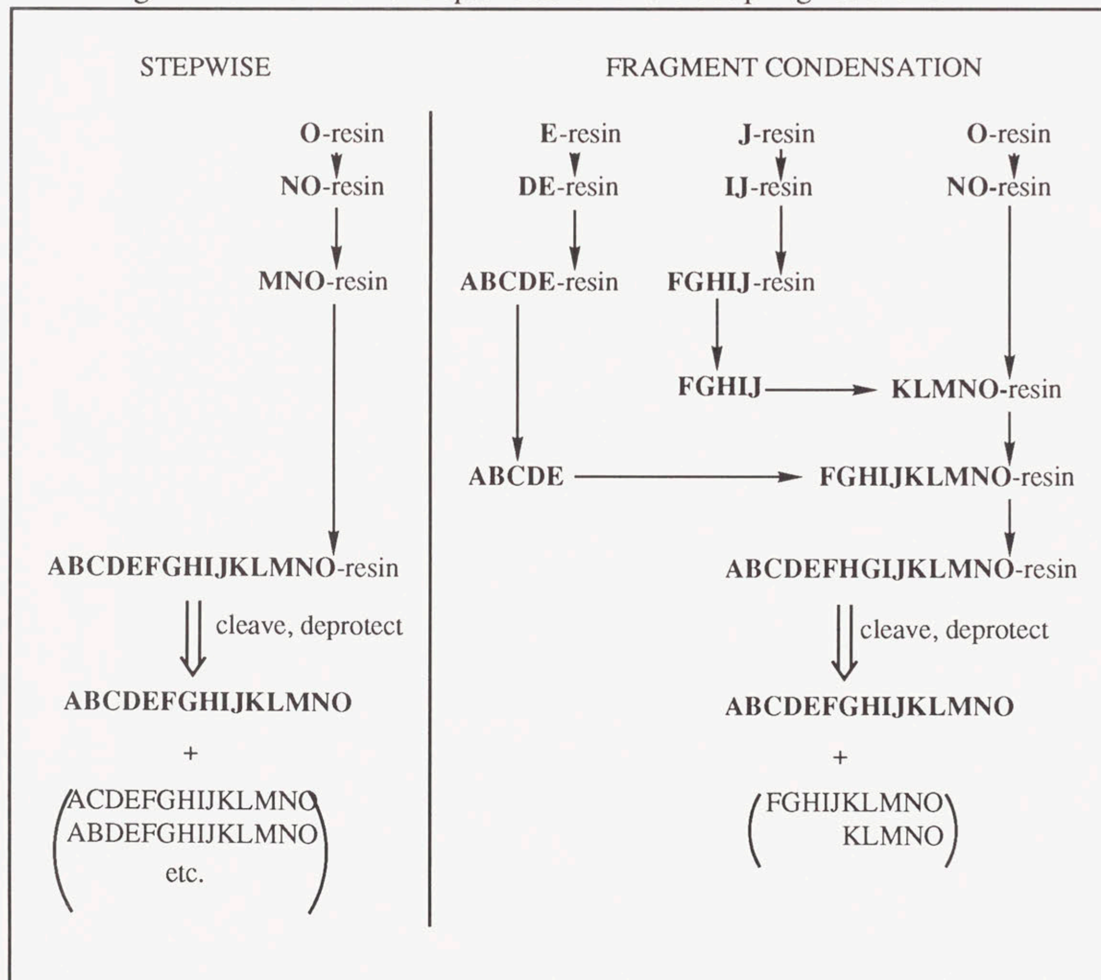


Figure 1. Stepwise vs. Fragment Condensation

Although several remarkable stepwise syntheses of larger peptides have been reported in the literature, such as the total synthesis of the HIV-1 protease (99 residues) by Schneider and Kent in 1988[7], .the synthesis and purification of peptides in a stepwise fashion can be prohibitvely difficult in peptides of greater than 20-30 amino acids. [1] However for the preparation of many peptides less than 20 residues in length, the

stepwise synthetic strategy is satisfactory. All automated synthesizers marketed today, rely on the success of the stepwise synthetic strategy.[5]

An alternative strategy to the stepwise assembly of a peptide is the fragment condensation method. The assembly of peptides in fragments was commonly employed in solution phase peptide syntheses, because of the relative ease of purifying the fragments.[2] The total synthesis of ribonuclease A reported by Yajima and Fuji in 1980 was achieved by solution phase fragment condensation [8]. Solid phase application of fragment condensations awaited the development of a resin from which one could remove the peptide without removing the side chain protecting groups. Two resins which allow the synthesis of protected peptides are the Kaiser resin for use with BOC-amino acids[9] and the SASRIN (super acid sensitive) resin for use with FMOC amino acids.[10] An advantage to fragment condensation, is that the peptide fragments are usually small (5-10 residues) and are more easily purified. The final peptide is assembled from a series of highly purified fragments. In theory, the products of any incomplete fragment couplings will have very different molecular weights and chromatographic properties, so the purification of the final peptide sequence will be simplified. In addition, if one were planning to prepare a large number of sequence analogs, or incorporate a rare or expensive amino acid, one need only remake the relevant fragment. The disadvantage of the fragment condensation method is that the fragment couplings tend to be slow and proceed at lower yields as compared to coupling single amino acid.[1] In the stepwise strategy, a difficult coupling might be driven forward by using a large excess of incoming residue. However, this is often not practical in a fragment coupling where the fragment is the result of considerable time and effort. The protected peptide fragments also tend not to be as soluble as individual amino acids in the requisite organic solvents. In addition to the possible low yields, the slow coupling time means that intramolecular non-coupling reactions may compete.[1] The most serious competing side reaction is racemization or more precisely, epimerization.[11]

At the outset of this project, the Lansbury lab was committed to furthering the methodology for solid phase fragment condensation syntheses that had been developed by E. T. Kaiser Although the model sequence chosen from Antithrombin III was only 17 amino acids long, a number of analogs were planned so that pursuing a fragment condensation synthesis was appropriate. Howver, difficulties were encountered in the deprotection of the fragment condensation products. After some time was spent trying to remedy this problem, the peptides were succesfully synthesized using the stepwise strategy. In the following sections the syntheses of the peptides using the two different strategies will be described.

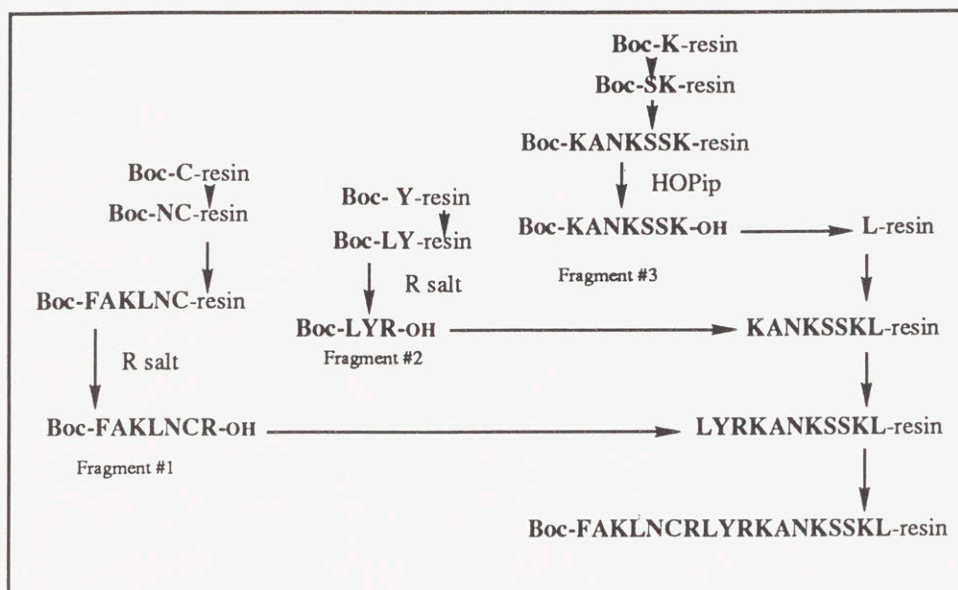
Synthesis of ATIII(123-139), ATIII Random and ATIII 2E via Fragment Condensation

The synthesis of peptides using the fragment condensation strategy necessitates a resin from which the peptide can be cleaved without removing the amino acid side chain protecting groups. In 1980 E.T. Kaiser reported the synthesis of a polystyrene based resin designed for the preparation of small peptides using BOC-amino acids.[9] The carboxyl terminus of an amino acid or peptideis linked to the resin via an oxime. Cleavage of the oxime-peptide linkage is achieved using nucleophiles (such as hydroxypiperidine or methyl amine). This method is entirely orthogonal to the acid-deprotection of the α–amino and side chain protecting groups. [12]

The Kaiser resin was not commercially available at the time this project was started. The resin was prepared in two steps from 1% crosslinked polystyrene-divinylbenzene.[13] First, Freidal-Crafts acylation using p-nitobenzoyl chloride was performed to generate the p-nitrobenzophenone. The ketone resin was then reacted with hydroxylamine hydrochloride to produce the oxime.

Each of the three peptides discussed so far (ATIII 123-139, ATIII Random and ATIII 2E) were to be assembled from three fragments. The middle fragment of each

peptide is the same, so 7 fragments were synthesized and assembled to generate the three peptides. (See Figure 2)



	#1	#2	#3	
ATIII Random	Boc-FKAKNCR	LYR	AKSSNLK	L-resin
ATIII 2E	Boc-FAKLNCE	LYR	KANESSK	L-resin

Figure 2. Construction of Peptide Fragments

The BOC (t-butoxycarbonyl) α–amino protected amino acids were used in the synthesis. Upon treatment with 25% TFA/CH_2Cl_2, the free amine is generated by acidolysis of the urethane ester bond followed by spontaneous decarboxylation. In preparing each fragment, the incoming amino acid was activated for coupling by preparing its symmetric anhydride using diisopropylcarbodiimide (DIC) just prior to addition to the reaction mixture. DIC is an analog of DCC (first demonstrated as an amino acid activating agent by Sheehan in 1955) where the urea byproduct is soluble in organic solvents.[14] The use of DIC eliminates a filtration step otherwise needed with DCC.

The protected peptide fragments were removed from the oxime resin by one of two methods. The first method employs the tetra n-butyl ammonium salt of an amino acid (the large counterion renders the free amino acid soluble in organic solvents) to

displace the peptide from the resin.[15] This method results in the addition of another amino acid to the C-terminus of the peptide. The second method employs N-hydroxypiperidine (HOPip)[16] The peptide is first released from the resin as the hydroxypiperidine ester. The HOPip ester is reduced to the free acid by reduction with Zn and acetic acid.

The synthesis and cleavage of the seven peptide fragments proceeded with few problems. The success of the syntheses and cleavages were monitored using quantitative amino acid analysis. The protected peptide fragments were purified on a semi-preparative reverse phase HPLC system. Their identity was verified using amino acid analysis, FABMS and ^{1}H-NMR spectroscopy. Final peptide yields after cleavage and purification ranged from 20-80% .

The three full protected peptides were assembled by coupling the requisite fragments to Lys(Cl-Z)-resin. See figure 2. Rather than using the symmetric anhydride of each fragment where one mole of fragment would be wasted with each mole of anhydride formed, the fragments were coupled as HOBt esters. The fragment coupling reactions were run at 4°C for the first hour of the approx. 48 hour reaction time to minimize racemization. HOBt also suppresses racemization.[17] N-methyl amine was used to cleave the full protected peptide from the resin leaving the carboxyl terminus modified as a methyl amide.[12]

Through out the syntheses, the amino acid side chains were protected with benzyl or benzyl derivatives, with the notable exception of the arginine guanidinium which was protected a nitro group. These groups were chosen because they could be removed without the use of an anhydrous HF line which was not available at the time. The lysine(Cl-Z), serine(Bzl) and tyrosine(Cl_2-Bzl) protecting groups were reported to be removable in trifuoromethane sulfonic acid (TFMSA).[18] The cysteine protecting group, 4-methyl benzyl , was presumed to also be removable using TFMSA, but later experience

and literature searching revealed that this was not the case.[19] Pd catalytic transfer hydrogenation using Pd Black and formic acid was reported to deprotect nitro-arginine.[20]

Before the full peptides were deprotected, a number of experiments were performed in order to develop a protocol for combining the catalytic transfer hydrogenation (Pd black/formic aicd) and the TFMSA deprotection methods. First, individual Boc amino acids were deprotected using Pd Black/ formic acid. All the amino acids used in these syntheses were deprotected within one hour, as monitored by TLC, except for Boc-Cys(4-methylbenzl). After five hours, the cysteine starting material was still unchanged. It was assumed that TFMSA would deprotect the Boc Cys (4-methyl benzyl) but this was never tested directly.

A deprotection scheme was devised by which the peptide would first be treated with Pd black/formic acid. The dried crude product of the catalytic hydrogenation treated directly with TFMSA. This "double deprotection" scheme was tested on Boc-FAK(Cl-Bzl)LNC(4-meBzl)R(NO_2)-OH since this fragment contained the potentially problematic protecting groups. Analytical HPLC of the crude deprotected peptide revealed a number of products. Two products positively identified by FABMS were the fully deprotected peptide and the Cys(4-meBzl) containing peptide. In the analytical HPLC trace, the correct product was the smallest peak. However, the quantity of the two identifiable products was not determined, nor were the remaining impurities identified.

Despite the evidence that deprotection methods chosen left a significant portion of the product with at least one remaining protecting group, each of the three full protected peptides were subjected to the "double deprotection" procedure. Analytical HPLC of the crude deprotection products revealed a multitude of peaks. The deprotection products were first subjected to gel filtration chromatography. Fractions from different regions of the gel chromatograph revealed a different array of peaks by HPLC. ^{1}H-NMR spectroscopy of the crude, post gel filtration material showed a large number of impurities. We could not determine whether the aberrant signals were the result of

unremoved protecting groups or scavengers. Both reverse phase and ionic exchange HPLC were employed in an attempt to isolate homogeneous products. Neither the HPLC products nor the crude post gel filtration material showed any identifiable deprotection products by FABMS.

At the same time as it became evident that the purification of the deprotection products was going to be extremely difficult, the opportunity to use a different synthetic strategy arose. A du Pont semi-automatic peptide synthesizer was offered to the Lansbury lab by Hoffman-LaRoche. The stepwise synthesis of the Anithrombin III peptides using the duPont apparatus is discussed in the next section.

Synthesis of ATIII(123-139) and ATIII Random in a Stepwise Strategy

The DuPont RaMPS synthesizer was designed for use with FMOC-amino acids. The α-amine of an FMOC-amino acid is protected with 9-fluorenylmethoxycarbonyl (FMOC). FMOC-amino acids were developed in an effort to eliminate the repeated acid treatment of the peptide which is otherwise necessary when BOC-amino acids are used.[21] Reports of acid sensitive peptide bonds as well as acid catalyzed side reactions prompted the development of either more acid labile α-amino protection (e.g. Bpoc or 2-(4-biphenyl)propyl[2]oxycarbonyl) or groups that could be removed using entirely orthogonal methods such as dithioasuccinyl (Dts)[22] which is removable via thiolysis or FMOC.[23] In addition, the peptides synthesized using FMOC-amino acids can be fully deprotected with TFA rather the more dangerous TFMSA or a hazardous anhydrous HF line. The first syntheses using FMOC amino acids were reported in 1978.[24]

Fmoc is a urethane protecting group which can be removed by a short treatment (~9 minutes) of either a primary or secondary amine.[21] In the synthesis of the peptides reported here, 50/50 (vol.) piperidine/DMF was used. The deprotection proceeds via a β-elimination of the fluorenylmethyl ester followed by spontaneous decarboxylation.

In the FMOC syntheses, two different coupling methods were employed. In the initial smaller scale syntheses, the incoming amino acid was added as a preformed pentafluorphenyl ester (Pfp-ester). Pfp-esters are sufficiently stable to be purified and crystallized.[25] The advantage of a purified active ester is that any side products of the activation step have been removed . Commercially available Pfp-esters were used in the smaller scale synthesis, but were too expensive for use in the larger scale preparations. In the second synthesis, the couplings were achieved using the BOP (Castros reagent) coupling reagent.[26] BOP is a derivative of HOBt that allows coupling reactions to proceed more rapidly and at higher yields than the more conventional DCC methods.[27] Since BOP couplings are run in DMF rather than CH_2Cl_2, fewer coupling problems due to poor solvation of the resin or reagents are expected.[5] The BOP reagent is used with DIEA to activate the amino acid *in situ* . After the lsat amino acid was coupled to the peptide chain, the FMOC group was removed and the free amino terminus was acetylated with acetic anhydride.

The basic cleavage conditions allow for side chain protection and a peptide-resin linkage which are relatively acid labile (90% TFA).[21] Serine, tyrosine and cysteine were protected as t-butyl ethers. Lysine was protected with BOC. Arginine was protected with Mtr(4-methoxy-2,3,6,trimethylbenzene-sulfonyl). Asparagine was left unprotected. The completed peptide was cleaved from the resin and deprotected using 90% TFA with 1,2-ethane dithiol and thiol anisole as scavengers.[28] The resin used, duPont Rapid Amide™, is a 2,4-dimethoxybenzhydralamine resin. Peptides are released from this resin with a C-terminal amide.[29]

In the initial syntheses of the peptides, cysteine was protected as the t-butyl thioether. The thioether is not cleaved under the 90% TFA conditions that cleave the other protecting groups, but can be removed in a separate step using mercuric (II) acetate.[30] Because the peptides were to be studied in aqueous solutions at a variety of pH's and buffer conditions, the cysteine was left protected, to avoid the possibility of dimerization.

The corresponding cysteine (Cys 128) in the native protein forms a disulfide bond with Cys 8.[31] Therefore the free sulfhydryl peptide is no more representative of the native protein than is the t-butyl thioether.

ATIII(123-139) and ATIII Random were each synthesized in a stepwise fashion at a 0.1 mmole scale using the duPont Rapid Amide Synthesizer. The major product was the correct product in both syntheses. ATIII(123-139) and ATIII Random were resynthesized on a 0.6 mmole scale using multiple aliquots of the RaMPs resin emptied into a standard glass synthesis vessel. During this second synthesis a complication arose, which was ultimately traced back to the fact that the Fmoc Cys(tBu) used in the synthesis actually possessed a different side chain protecting group than the label of the bottle indicated. There were two major products in the second synthesis of ATIII(123-139). One product had a FABMS and a ^{1}H-NMR consistent with the free sulfhydryl peptide, suggesting that the thioether group had indeed been removed by 90% TFA. The second product had a FABMS that was high by 32 mass units, but a ^{1}H-NMR spectrum virtually identical to the correct t-butyl thioether peptide product. A thorough investigation of theses two products revealed that the M+ 32 peak contained a protecting group called Cys t-butyl mercapto or Cys(S-S-tbutyl) Upon treatment of the M+32 peptide product with DTT, a new peak appeared on analytical HPLC which had a FABMS corresponding to the free sulfhydryl peptide.[32]

The bottle containing the Fmoc Cys used in the large scale syntheses had since been emptied and discarded. However, another bottle labeled with both Cys(tbutyl) and the MW 399.5 corresponding to the thioether, was shown by FABMS to actually contain a compound with MW 431.6 corresponding to the t-butyl mercapto protecting group. If one compares various catalogs selling FMOC amino acids, one finds that the term Fmoc (S-tbutyl) is used to mean t-butyl thioether by some and t-butyl mercapto- by others. This complication accentuates the importance of fully characterizing the peptide products. This change in Cys protecting groups would not have been detected by

sequencing or amino acid analysis nor even ^{1}H-NMR. All the experiments described in Chapters 4 and 5 of this thesis, employed peptides with the Cys(S-S-tbutyl) protecting group.

Synthesis of ATIII N135 GlcNAc

The sequence 123-139 of human Antithrombin III encompasses a glycosylation site at Asn 135. One report in the literature suggested that the heparin binding capacity of ATIII was higher in a natural variant of the protein lacking the carbohydrate moiety at Asn 135.[33] Since the technology for prparing small glycopeptides was available in the Lansbury laboratory, we thought it would be interesting to prepare a glycosylated version of the ATIII(123-139) peptide and compare its behavior to that of ATIII(123-139).

Glycopeptide synthesis is challenging because the carbohydrates one would like to attach to a peptide are often either difficult to synthesize or laborious to isolate from natural sources. The glycosidic linkages of oligosaccharides can be acid labile and therefore incompatible with conditions used in peptide synthesis.[34] To generate a glycopeptide bearing only a one or two saccharide side chain, it can be sufficient to prepare a glycosylated amino acid for use directly in the synthesis. [35]

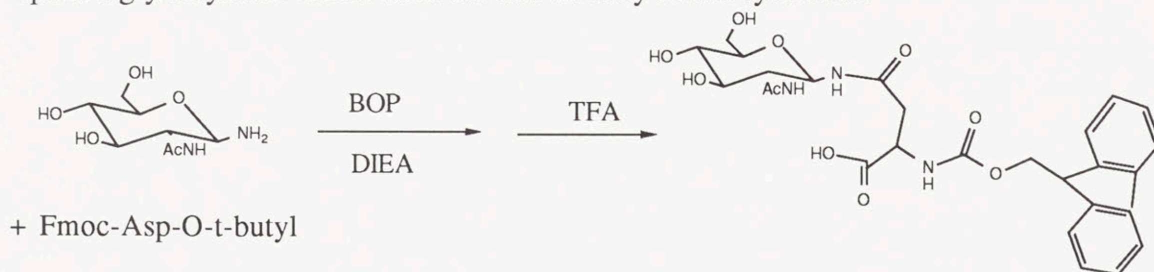


Figure 3. Coupling N-acetylglucosamine to FMOC Asp-αO-t-butyl

For the purposes of this project, a single N-acetyl glucosamine (GlcNAc) attached to asparagine was sufficient to initiate the study. The glycopeptide was prepared by linking N-acetyl glucosamine to the side chain carboxylate of Fmoc-Asp.[35](See figure 3). The reaction was performed using the BOP reagent in conditions very similar to those used to make a peptide bond.[36] This reaction generates exclusively the β-anomeric linkage. This linkage configuration is found naturally in glycoproteins. The resulting

Fmoc Asn(GlcNAc) was used directly in the peptide synthesis instead of Fmoc-Asn. The use of any acetylating agents was avoided after the Fmoc-Asn(GlcNAc) had been coupled.

The purification of the product proved to be difficult because correct glycopeptide had very similar HPLC elution behavior as the product missing the Cys t-butyl and a product missing the GlcNAc. The correct material was purified in two different HPLC purification rounds. The final product gave no evidence of the contaminating side products in the plasma desorption mass spec. In the ^{1}H-NMR spectrum of the product, singlets from the peptide amide terminal acetyl group and the N-acetyl glucosamine acetyl group integrated to equal values, indicating that all the isolated peptide bore a glucosamine residue.

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Chapter 3. Experimental Methods

Equipment, Materials, and Supplies

All protected FMOC amino acids were purchased from DuPont, Bachem or Novabiochem. BOP was purchased from Richelieu. The Rapid Amide resin was obtained in individual cartridges from DuPont. Diisopropylethylamine was purchased from Aldrich Chemical Co. and distilled over ninhydrin immediately before use. Dimethylformamide (DMF) was purchased from Baxter. All other solvents used were HPLC grade.

HPLC was performed on a Waters 600 HPLC system equipped with either a 481 single wavelength detector or a Waters 994 Diode Array Detector. C_4-300A reverse phase columns (analytical-1.9 x 30 cm, semi-preparative-19 x 30 cm) were purchased from either Waters or Vydac. HPLC grade acetonitrile was purchased from J.T. Baker. TFE and TFA were purchased from Aldrich. The water was purified on a Waters Milli-Q purification system.

Amino acid analysis was performed on a Waters 501 Picotag amino acid analyzer. Analytical grade 6N HCl, 50/50 HCl/propionic acid, and PITC were purchased from Pierce. Ultra-pure Gdn-HCl was purchased from ICN.

Fast Atom Bombardment Mass Spectrometry (FAB-MS) was performed by Dr. Iaonnos Papayanopoulos of Professor Klaus Biemen's laboratory and Dr. Andrew Tyler of the Harvard University Chemistry Department Mass Spectrometry Laboratory. Plasma Desorption Mass Spectrometry (PDMS) was performed by Mr. Marty Lacey of the Proctor and Gamble Co. or Mr. Edward Takach at the MIT Chemistry Department Spectroscopy Laboratory. ^{1}H-NMR spectra were run on a Varian 300 MHz. Deuterated solvents were purchased from either Cambridge Isotope Labs. or MSD solvents. Amino acid analysis was performed by the Biopolymers Laboratory at the MIT Cancer Research Center under the direction of Dr. Richard Cook.

<u>General Procedure for Peptide Synthesis using Protected Peptide Fragments</u>

Synthesis of the Kaiser p-Nitrobenzophenone Oxime Resin

p-Nitrobenzophenone resin (ketone-resin): Polystyrene-divinyl benzene (Biorad Biobeads S-X1, 200-400 mesh, 100 gms) was placed in a 2L flask (fitted with an overhead stirrer) and swelled with CH_2Cl_2 (1L). To the resin was added 4-nitrobenzoyl chloride (17.1 gms). The resin mixture was blanketed with Ar. Aluminum chloriude (17.1 gms) was added and the mixture briefly stirred. The reaction mixture turned bright orange. The mixture was allowed to sit under Ar for 24 hours with slow stirring. The resin was filtered away from the reaction mixture and washed with dioxane/3N aqu. HCl (3:1,4x), dioxane/H_2O (3:1,3x), DMF (3x), methanol (3x), CH_2Cl_2 (2x), and methanol (2x). The resin was dried *in vacuo*.

p-Nitrobenzophenone Oxime Resin: The p-Nitrobenzophenonoe resin from the previous step was added to a boiling solution of hydroxylamine hydrochloride (100 gm) in pyridine/ethanol (1:5,1L). The resin reaction mixture was refluxed under Ar for 20 hours. The resin was filtered from the reaction mixture and washed with methanol/H2O (3:1,4x), DMF (2x), CH_2Cl_2 (2x), methanol (2x), CH_2Cl_2 (2x), and methanol (3x). The resin was dried *in vacuo*. A small quantity of resin was coupled with a Boc-amino acid and the substitution level determined by quantitative amino acid analysis.

Coupling the C-terminal Amino Acid to the Oxime Resin.

An known quantitiy of oxime resin (approx. 0.8 mmol oxime/ gm resin) was suspended in a solution of Boc amino acid (1.5 equivs.) and diisopropylcarbodiimide (DIC, 2 equivs.) in CH_2Cl_2 (10 ml/gm). The suspension was shaken for 24 hours at room temperature. The resin was filtered , washed thoroughly with CH_2Cl_2, ethanol, CH_2Cl_2, and dried *in vacuo*. The substitution level of the resin was determined by quantitative amino acid analysis. Typically, values of 0.4 mmoles/g resin were obtained.

General Procedure for Synthesis of Protected Peptides

The Boc-amino acid resin (approx. 4 mmole/gm) was placed in a glass synthesis vessel and swollen with CH_2Cl_2. The resin was rinsed once with CH_2Cl_2 and suspended in CH_2Cl_2 (15 ml/gm), acetic anhydride (10 equiv), and diisopropylethylamine(DIEA, 5 equivs). The reaction mixture was shaken for 6-12 hours at room temperature and rinsed with CH_2Cl_2. The following procedure was employed for the addition of each amino acid to the resin: (1) wash with CH_2Cl_2 (15 ml/gm, 2x1 min) (2) wash with 25% TFA in CH_2Cl_2 (15 ml/gm, 1x1 min), (3) shake with 25% TFA in CH_2Cl_2 (15 ml/gm, 1x30 min), (4) wash with CH_2Cl_2 (15 ml/gm, 2x1 min), (5) wash with isopropanol (15 ml/gm, 2x1 min), (6) wash with CH_2Cl_2 (15 ml/gm, 2x1 min), (7) wash with isopropanol (15 ml/gm, 2x1 min), and (8) wash with CH_2Cl_2 (15 ml/gm, 4x1 min). (9) The Kaiser test was performed on a small number of resin beads. The beads should turn bright blue indicating that the deprotection was succesful. (10) The symmetric anhydride of the incoming amino acid was prepared by combining 6.5 equivs. of Boc amino acid (based on equivs. amino acid on the resin) and 3 equivs. DIC in DMF. The mixture was allowed to stir for a few minutes and was added to the resin. (11) The resin shook briefly before adding 2 equivs. of DIEA. (12) The resin shoke for approx. 1 hour. at room temp. (13) wash with CH_2Cl_2 (15 ml/gm, 3x1 min). (14) wash with 33 % methanol in CH_2Cl_2 (15 ml/gm, 3x1 min). (15) wash with CH_2Cl_2 (15 ml/gm, 3x1 min). (16) Perform the Kaiser test on small number of resin beads. If yellow, indicating a complete coulpling reaction, one may either stop here or return to step (2) to prepare for the addition of another amino acid. If the result is blue or light blue, first rewash the resin and repeat the Kaiser test. If the results are still unsatisfactory, return to step (10) and repeat the coupling reaction.

Cleavage of the Peptide From the Resin Using N-Hydroxypiperidine.

To a suspension of resin-bound peptide in CH_2Cl_2 (10 ml/g, approx. 0.3 mmol peptide) was added N-hydroxypiperidine (HOPep, 2 euqiv.) in CH_2Cl_2 ([HOPip]=0.2M). The suspension was stirred vigorously for 12 hours at room temperature. The resin was

filtered, and washed with CH_2Cl_2, DMF, and methanol. The filtrate was concentrated to an oil and dissolved in 90% acetic acid/water (30 ml/mmol peptide). Zinc dust (10 equivs) was added and the suspension stirred for 1 hour at room temperature. The suspension was filtered and, upon addition of water (3-6 volume equivs.), a white precipitate came out of solution in most cases. The precipitate was isolated by centrifugation and washed several times with water. The filtrate was saved and if the yield was low, reprecipitated. If a precipitate does not form, the aqueous filtrate (containing zinc acetate) can be lyopholized and used directly for further purification.

Preparation of Amino Acid Tetra N-Butyl Ammonium Salts

To a suspension of amino acid in 1:1 methanol-water (0.3 M) was added tetra N-butyl ammonium hydroxide (1.54 M in H_2O, 0.95 equivs). The suspension cleared within 10 minutes. After stirring for 1 hour at room temperature, the methanol was removed b evaporation and the resultant aqueous solution was lyopholized to yield a clear gel. The gel can be used directly, without further purification, for the cleavage reactions.

The tetra N-butyl ammonium salts of Boc Glu and Boc Asp benzyl esters cannot be prepared, as the side chain protecting groups of these amino acids are lost during salt preparation.

Cleavage of Peptide-Resin Using the Tetra N-Butyl Ammonium Salts

A solution of amino acid tetra n-butyl ammonium salt (2 equiv) in DMF (0.1 M) was added to resin-bound peptide (0.2-0.4 mmole/gm) swollen with CH_2Cl_2. The reaction mixture was stirred for 24 hours at room temperature, then filtered to remove the resin. The resin was washed with CH_2Cl_2, methanol and DMF and the combined filtrate was concentrated to give a yellow oil. The oil was dissolved in a minimal amount of DMF (0.5 ml/0.1 mmol peptide) and 10 % aqueous citric acid was added to precipitate protected peptide. The fluffy white precipitate was isolated by centrifugation and washed

thoroughtly with water. Quantitative amino acid analysis can be performed on both the cleaved resin and the product in order assess the degree of completion of the reaction. The crude peptide was subsequently purified using either gel filtration or HPLC chromatography.

Synthesis of Protected Peptide Fragments

ATIII(123-139) Fragment #1 : Boc-FAK(Cl-Z)LNC(4-me-Bzl)R(NO_2)-COOH:

The peptide was synthesized on a 1 mmole scale using the standard procedure startng with resin-bound Cys(4-me-Bzl). The protected peptide was cleaved from the resin using Arg(NO2) tetra N-butyl ammonium salt. The crude peptide product was initially purified on an LH-20 gel filtration column in DMF. The correct peptide product was isolated from a semi-preparative HPLC gradient of 55/45 to 20/80 in 20 minutes. The major peak was the correct product. The final yield was 39% based on initial resin bound cysteine. Amino acid analysis: F 0.62 (1 expected) A 1.0(1) K 0.77(1) L 1.2(1) N 1.3(1) C - R 1.0(1). FAB-MS: 1290 $(M+Na)^+$. Analytical HPLC: retention volume 32 mls at 54/46 H_2O/CH_3CN + 0.1% AcOH. ^{1}H-NMR (d_6-DMF, 300 MHz) δ 8.3-7.9(m),:7.5-6.9 (m), 5.1 (s, 2H, Lys Bzl), 4.6-4.1 (m), 3.7 (s, 2H, Cys Bzl), 3.1 (m), 2.9 (m), 2.8-2.3 (m), 2.2 (s, 3H, Cys Bzl-CH_3) 1.3 (s, 9H, BOC δ-CH3), 0.8 (m, 6H, Leu δ-CH_3)

ATIII(123-139) Fragment #2: Boc-LY(Cl_2-Bzl)R(NO_2)-COOH

The peptide was synthesized on a 1 mmole scale using the standard procedure startng with resin-bound Tyr(Cl_2-Bzl). The protected peptide was cleaved from the resin using Arg(NO_2) tetra N-butyl ammonium salt. The crude peptide product was purified using semi-preparative HPLC at isocratic conditions 44/56 H_20/CH_3CN + 0.1% AcOH. The final yield was 88% based on resin bound Tyr. Amino acid analysis: L 1.5(1) Y

1.0(1) R 1.2(1). FAB-MS: 754(M+H)+, 776 (M+Na)+ Analytical HPLC: ^{1}H-NMR (d_6-DMF, 300 MHz) δ 8.3 (d), 8.2 (d), 8.1 (d), 7.7 (d), 7.6-7.4 (m), 7.2 (d, 2H, Tyr 2,6,H), 6.9 (d, 2H, Tyr 3,5 H), 5.1 (s, 2H Tyr Bzl), 4.6 (m, 1H, Tyr α-CH), 4.2 (m, 1H, ? α-CH), 3.8 (m,1H, ? α-CH), 3.2 (m, 2H, Arg δ-CH_2), 1.8 (m), 1.5 (m), 1.3 (s, 9H, BOC tert-butyl), 0.8 (m, 6H, Leu δ-CH_3)

ATIII(123-139) Fragment #3: Boc-K(Cl-Z)ANK(Cl-Z)S(Bzl)S(Bzl)-COOH

The peptide was synthesized on a 1 mmole scale using the standard procedure startng with resin-bound Ser(Bzl). The protected peptide was cleaved from the resin using N-hydroxypiperidine. The crude peptide product was initially purified on an LH-20 gel filtration column in DMF. The correct peptide product was isolated from a semi-preparative HPLC gradient of 40/60 to 0/100 in 15 minutes. The major peak was the correct product. The final yield was 15% based on initial resin bound Ser. One cause for the low yield is that the peptide stuck to the resin after cleavage with HOPip and could was not recovered unless the resin was washed with copius amounts of DMF. Amino acid analysis: K 0.8(2 expected) A 1.0(1) N 1.0(1) S N.A.. FAB-MS: 1250 $(M+H)^+$, 1273 $(M+Na)^+$. ^{1}H-NMR (d_6-DMF, 300 MHz) δ 8.3 (m), 8.2 (d), 7.9 (d), 7.8 (d), 7.5-7.3 (m, Lys and Ser aromatic), 6.9 (m), 5.1 (s, 4H, Lys Bzl), 4.7-4.4 (m), 4.4 (s, 4H, Ser bzl)4.3 (m), 3.9 (m), 3.8 (m), 3.7 (m), 3.6 (m), 2.6-2.4 (m, Asn β H), 1.3 (s, 9H, Boc tert-butyl)

ATIII Random Fragment #1 :Boc-FK(Cl-Z)AK(Cl-Z)NC(4-me-Bzl)R(NO_2)-COOH

The peptide was synthesized on a 1 mmole scale using the standard procedure startng with resin-bound Cys(4-me-Bzl). The protected peptide was cleaved from the resin using Arg(NO_2) tetra N-butyl ammonium salt. The crude peptide product was initially purified on an LH-20 gel filtration column in DMF. The correct peptide product was isolated from a semi-preparative HPLC gradient of 50/50 to 20/80 in 15 minutes.

The major peak was the correct product. The final yield was 39% based on initial resin bound cysteine. Amino acid analysis: FAB-MS: 1455$(M+H)^+$, 1477 $(M+Na)^+$. ^{1}H-NMR (d_6-DMF, 300 MHz): δ 8.2-7.9 (m), 7.5-6.9 (m), 5.1 (s,4H, Lys bzl), 4.6-4.4 (m), 4.3-4.1 (m), 4.6 (s,2H,Cys bzl), 3.2 (m), 2.9 (m) 2.2 (s, 3H, Cys me-bzl-CH_3) 1.3 (s, Boc tert-butyl)

ATIII Random Fragment #2:

See ATIII(123-139) Fragment #2

ATIII Random Fragment #3Boc-AK(Cl-Z)S(Bzl)S(Bzl)NL-COOH:

The peptide was synthesized on a 1 mmole scale using the standard procedure startng with resin-bound Leu. The protected peptide was cleaved from the resin using N-hydroxypiperidine. The crude peptide product was initially purified on an LH-20 gel filtration column in DMF. The correct peptide product was isolated from a semi-preparative HPLC gradient of 45/55 to 35/65 in 10 minutes. The major peak was the correct product. The final yield was 30 % based on initial resin bound Leu. Amino acid analysis: A 1.0(1 expected) K 0.68(1) S 0.71(2) N 2.3(1) L 3.1(1) ^{1}H-NMR (d_6-DMSO, 300 MHz) 8.3 (d), 8.2 (t), 7.9 (d), 7.8 (d), 7.5-7.2 (m, Lys and Ser aromatic), 5.1 (s, Lys bzl), 4.4 (s, Ser bzl), 4.1 (m), 3.9 (m), 3.6 (m), 2.9 (m) 2.6-2.4 (m), 1.3 (s, Boc-tert butyl), 1.1 (d, Ala β-CH3), 0.8 (m,Leu,δ CH3)

ATIII 2E Fragment #1Boc-FAK(Cl-Z)NC(4-me-Bzl)E(Bzl)-COOH:

The peptide was synthesized on a 1 mmole scale using the standard procedure startng with resin-bound Glu. The protected peptide was cleaved from the resin using N-hydroxypiperidine. The crude peptide product was initially purified on an LH-20 gel filtration column in DMF. The correct peptide product was isolated from a semi-preparative HPLC gradient of 42/58 to 12/88 in 15 minutes. The major peak was the

correct product. The final yield is unkown. Amino acid analysis: F 0.87 (1 expected) A 1.2(1) K 0.81(1) L 1.0(1) N 0.77(1) C - E 1.2(1). FAB-MS: 1274$(M+H)^+$, 1292$(M+Na)^+$ ^{1}H-NMR (d_6-DMF, 300 MHz) d 8.2-7.8 (δ, α-NHs), 7.5-7.0 (m, Lys, Cys, Glu aromatic) 5.1 (s, 4H, Lys and Glu bzl), 4.6-4.0 (m,α-CH), 3.6 (s, 2H, Cys bzl), 3.1 (m), 2.8-2.4 (m), 2.3 (s,3H, Cys me-bzl CH_3), 1.3 (BOC ter-butyl) 1.2 (m, Ala β-CH_3), 0.8 (m, 6H, Leu δ-CH_3)

ATIII 2E Fragment #2:

See ATIII(123-139) Fragment #2

ATIII 2E Fragment #3Boc- K(Cl-Z)ANE(Bzl)S(Bzl)S(Bzl)-COOH:

The peptide was synthesized on a 1 mmole scale using the standard procedure startng with resin-bound Ser(Bzl). The protected peptide was cleaved from the resin using N-hydroxypiperidine. The crude peptide product was initially purified on an LH-20 gel filtration column in DMF. The correct peptide product was isolated from a semi-preparative HPLC gradient of 38/62 to 20/80 in 10 minutes. The major peak was the correct product. The final yield was 20% based on initial resin bound Ser. Amino acid analysis: K 0.96(2 expected) A 0.97(1) N 1.2(1) E 1.4(1) S 1.0(2) FAB-MS: 1174 $(M+H)^+$, 1197 $(M+Na)^+$. ^{1}H-NMR (d_6-DMF, 300 MHz) [spectrum shown in Appendix A is on the wrong scale] 7.9 (t), 7.6-7.4 (m, Lys, Glu, and Ser aromatics), 5.1 (2s, 4H, Lys and Glu bzl), 4.6 (m), 4.4 (s, Ser bzl), 4.3 (m), 4.2 (m) 3.9 (m) 3.8 (m) 3.7 (m) 3.0 (m) 1.3 (BOC tert-butyl), 1.2 (m,Ala β-CH_3)

General Procedure for Solid Phase Coupling Protected Fragments

An aliquot of resin bound Boc-amino acid (ca. 1.0 gm, 0.3 mmole peptide) wsa placed in a small synthesis vessel and swelled with CH_2Cl_2. Acetic anhydride (1.5 mmole) and N-methyl morpholine (0.75 mmole) were added to the suspension and

allowed to shake for 8 hours at room temperature. The resin was washed thoroughly with CH_2Cl_2 (10 ml/gm, 3x1 min.). The resin was rinsed briefly with 25% TFA/CH_2Cl_2, (10 ml/gm, 1x1min.and then shaken in for 30 minutes in 25% TFA/CH_2Cl_2. After deprotection the resin was rinsed with CH_2Cl_2 (10 ml/gm, 3x1 min.), isopropanol (10 ml/gm, 3x1 min.) and CH_2Cl_2 (10 ml/gm, 3x1 min.). The resin was then neutralized by washing thoroughly with 1% NMM/CH_2Cl_2 (10 ml/gm, 5x1 min.) and rinsed again with CH_2Cl_2 (10 ml/gm, 3x1 min.). The entire synthesis vessel, with the resin swelled in minimal CH_2Cl_2, was transfered to a 4°C cold room or cold box. The "incoming fragment" was dissolved in minimal DMF (0.075 mmole, 3 mls.), chilled to 4°C, and add to the resin. DIC (0.2 mmole, 0.05M in DMF) and HOBT (0.2 mmole, 1 M in DMF) were in eached cooled to 4°C and added to the resin. The reaction mixture was shaken gently for 1 hour at 4°C. The mixture was then allowed to shake for 24-48 hours at room temperature. Aliquots of resin were removed, rinsed, and dried for use in quantitative amino acid analysis at once or twice during the coupling. Additional aliquots of DIC were sometimes added after the reaction had run for approx, 12 hours. Once successful coupling had been verified by amino acid analysis, the resin was filtered and rinsed with DMF, CH_2Cl_2, and isopropanol. The coupling supernatant may be saved for analysis or recycling of the unreacted peptide fragment. The resin was acetylated as described earlier in preparation for addition of the next fragment.

Assembly of Full Peptides From the Protected Fragements

Synthesis of ATIII(123-139)-resin:

Coupling KANKSS to K-resin: Lys(Cl-Z)-resin (1.0 gm, 0.29 mmoles) was acetylated, deprotected, neutralized (as described in the general procedures) and coupled with Boc-K(Cl-Z)ANK(Cl-Z)S(Bzl)S(Bzl)-OH (0.075 mmoles) using DIC (0.1 mmole) in the

presence of HOBt (0.2 mmole) for 48 hours. Coupling yield as determined by amino acid analysis was 17 %.

Coupling LYR to KANKSSK-resin: KANKSSK-resin was acetylated,deprotected, neutralized and coupled with Boc-LY(Cl_2-Bzl)R(NO_2)-OH (0.07 mmoles) using DIC (0.1mmole) in the presence of HOBt (0.2 mmole) for 48 hours. The coupling was quantitative as determined by amino acid analysis .

Coupling FAKLNCR to LYRKANKSSK-resin: LYRKANKSSK-resin was acetylated,deprotected, neutralized and coupled with Boc- FAK(Cl-Z)LNCR(NO_2)-OH (0.06 mmoles) using DIC (0.1mmole) in the presence of HOBt (0.2 mmole) for 48 hours. The coupling was quantitative as determined by amino acid analysis .

Final amino acid analysis of the resin bound peptide:

F 0.73 (1 expected) A 2.3 (2) K 4.4 (4) L 1.9(2) N 2.1(2) C- R 1.0 (2) Y 0.82 (1) S 1.1 (2)

0.80 gms of resin were recovered at 0.021 mmoles peptide/gm (based on Y)

Synthesis of ATIII Random-resin:

Coupling AKSSNL to K-resin: Lys(Cl-Z)-resin (1.6 gm, 0. 46 mmoles) was acetylated, deprotected, neutralized (as described in the general procedures) and coupled with Boc-AK(Cl-Z)S(Bzl)S(Bzl)NL-OH (0.08 mmoles) using DIC (0.1 mmole) in the presence of HOBt (0.2 mmole) for 28 hours. Coupling yield as determined by amino acid analysis was 40 %.

Coupling LYR to AKSSNLK-resin: AKSSNLK-resin was acetylated,deprotected, neutralized and coupled with Boc-LY(Cl_2-Bzl)R(NO_2)-OH (0.12 mmoles) using DIC (0.1mmole) in the presence of HOBt (0.2 mmole) for 25 hours. Coupling yield as determined by amino acid analysis was 66 %.

Coupling FKAKNCR to LYRAKSSNLK-resin: LYRAKSSNLK-resin was acetylated, deprotected, neutralized, and coupled with Boc- FK(Cl-Z)AK(Cl-Z)NCR(NO_2)-OH (0.08 mmoles) using DIC (0.22mmole) in the presence of HOBt (0.4 mmole) for 51 hours. Coupling yield as determined by amino acid analysis was > 100%.

Final amino acid analysis of the resin bound peptide:

F 3.3(1 expected) A 0.83(2) K 3.8(4) L 1.4(2) N 1.3(2) C- R 0.45(2) Y 1.0(1) S 0.29(2)

1.4 gms of resin were recovered at 0.024 mmoles peptide/gm (based on Y)

Synthesis of ATIII 2E-resin:

Coupling KANESS to K-resin: Lys(Cl-Z)-resin (1.6 gms, 0.45 mmoles) was acetylated, deprotected, neutralized (as described in the general procedures) and coupled with Boc-K(Cl-Z)ANE(Bzl)S(Bzl)S(Bzl)-OH (0.09mmoles) using DIC (0.1 mmole) in the presence of HOBt (0.2 mmole) for 46 hours. The coupling was quantitative as determined by amino acid analysis .

Coupling LYR to KANESSK-resin: KANESSK-resin was acetylated,deprotected, neutralized and coupled with Boc-LY(Cl_2-Bzl)R(NO_2)-OH (0.12 mmoles) using DIC (0.1mmole) in the presence of HOBt (0.2 mmole) for 44 hours. Coupling yield as determined by amino acid analysis was 37 %.

Coupling FAKLNCEto LYRKANESSK-resin: LYRKANESSK-resin was acetylated,deprotected, neutralized and coupled with Boc- FAK(Cl-Z)LNCE(Bzl)-OH (0.05 mmoles) using DIC (0.1mmole) in the presence of HOBt (0.2 mmole) for 44 hours. Coupling yield as determined by amino acid analysis was 70 %.

Final amino acid analysis of the resin bound peptide:

F 0.91 (1 expected) A 2.2(2) K 3.4 (3) L 2.2 (2) N 1.6 (2) C- E 1.8 (2) R 0.6 (1) Y 1.0 (1) S 1.1 (2)

1.4 gms of resin were recovered at 0.011 mmoles peptide/gm (based on Y)

"Double" Deprotection Procedure

Catalytic transfer hydrogenation was achieved by dissolving the dry crude peptide (3-18 umoles) in 2 mls. of 88% formic acid and adding 60 mgs. of Pd Black. The reaction stirred for 24 hours after which time the Pd was removed by centrifugation. The Pd was washed 2x with 500 uls formic acid. The formic acid from the reaction and the washes was pooled and removed under vacuum. To the dried crude product on ice was added 100 uls. thioanisole, and 50 uls. 1,2 ethanedithiol. 1.0 ml neat TFA was added and the reaction stirred for 10 min. 100 uls. of TFMSA was aded dropwise and the reaction stirred for an additional 30 mins. on ice. The product was precipitated from cold ether, and washed 3x with more cold ether. The crude product was isolated via centrifugation.

General Procedure for Stepwise Peptide Synthesis

Stepwise Synthesis of Peptides Using the FMOC-Amino Terminus Protection Scheme

The resin was placed in a glass reaction vessel and rinsed with methylene chloride (15 ml./gm, 3x 1 min.) and DMF (15 ml./gm, 3x 1 min). The following procedure was used for the addition of each amino acid to the resin or the growing peptide chain: (1) Wash the resin with DMF (15 ml/gm, 3x 1 min.), (2) wash with 50/50 (vol.) piperidine/DMF (15 ml/gm, 1x 1 min), (3) shake the resin in 50/50(vol.) piperidine/DMF (20 ml/gm, 7 min.), (4) wash with DMF (15 ml/gm, 3x 1 min.), (5) wash with methylene chloride (15 ml./gm, 3x 1 min), (6) wash with DMF (15 ml./gm, 3x 1 min), (7) add FMOC-amino acid (3 equivs., 0.1 M in DMF) and BOP (3 equivs., 0.1M in DMF), shake resin for 30 secs., (8) add DIEA (5.3 equivs.), shake mixture for 1 hour at room

temperature, (9) wash with DMF (15 ml./gm, 3x 1 min), (10) wash with methylene chloride (15 ml./gm, 3x 1 min), (11) perform the Kaiser test for free amine on a few beads of washed resin: if positive (blue), repeat steps (7)-(11) until a negative (yellow) result is obtained. After coupling the final residue, the amino-terminus was deprotected and acetylated with 10 equivalents of acetic anhydride and 5 equivalents of DIEA in methylene chloride for 30 minutes.

General Cleavage and Deprotection Procedure for FMOC Strategy Peptides

To the resin bound peptide was added the following mixture:

90 % (v/v) TFA

5 % (v/v) 1,2,ethanedithiol

4 % (v/v) water

1 % (v/v) thioanisole

A 4 ml. mixture was sufficient to deprotect ~ 100 umoles of resin bound peptide. This reaction was left to stir for 12-36 hours. The resin was filtered and washed with minimal TFA. The filtrate and TFA wash was combined and reduced to ~1 ml. under a stream of N_2(Ar) Crude deprotected peptide was precipitated from the concentrate and washed 3x, using cold diethyl ether.

Preparation of FMOC-Asn(GlcNAc)-OH

N-acetyl Glucosaminylamine: 450 mgs. (ca. 2 mmoles) of N-acetyl glucosamine was dissolved in 30 mls. H2) saturated with NH4 CO3. This solution stirred for 1 week, with periodic addition of NH4CO3 to maintain a saturation. Progress of the conversion of GlcNAc to GlcNAc-amine was monitored using silica TLC (4 EtOAc: 3 MeOH: 1 H2O: 0.1 TEA) stained with 1:1 20% aqu. H_2SO_4: 0.2 % naphthoresorcinol/EtOH. The product was isolated by repeated lyopholization of the reation mixture from H2O until no change in weight of the product was recorded (6-7 cycles of lyopholization from H2O). The product was used directly without further purification.

Preparation of FMOC-Asn(GLcNAc)-OH: FMOC-Asp-α–O-t-butyl ester was dissolved in a minimal quantity of DMF (1 equiv, 1 mmole/ml DMF) to which was added HObt (1 equiv, 2mmoles/ml DMF), HBTU (3 equivs, 0.6 mmoles/ml DMF), DIEA (1 equiv.), and N-acetyl glucsylamine amine (1 equiv., 2mmoles/3 mls. DMF). The reaction stirred for 20 hours at room temperature. The reafction mixtrue was then evaporated to a white solid. The solid product was washed with cold diethyl ether (20 mls., 2x) and cold H_2O (20 mls, 2x). The preoduct was recovered by centrifugation. A small aliquot of solid was used for analytical HPLC (100/0 to 30/70 in 20 mins., H_2O/CH_3CN + 0.1% TFA) to be compared with the strating material. > 100 % yeild was recovered by weight. No starting material was visible in the HPLC of the crude reaction product.

Lyopholized FMOC-Asn(GlcNAc)-α-O-t-butyl was dissolved in 10 mls. neat TFA. After stirring for 10 minutes, the TFA was removed under an Ar stream, The deprotected amino acid product was precipitated from cold ether, washed 2x with cold ether and recovered by centrifugation. The crude product was dissolved in DMF and puridfied by semi-prepartive HPLC (isocratic, 77/23,H_2O/CH_3CN + 0.1% TFA) The acetyl singlet and anomeric protons of the carbohydrate were clearly visible in the ^{1}H-NMR of the product. The coupling constant of the anomeric proton was 9 Hz, indicating that only the β-anomer was present. The HPLC purified product was used directly in peptide synthesis.

STEPWISE-SYNTHESIS OF PEPTIDES

ATIII (123-139)

Acetyl-FAKLNC(t-butyl)RLYRKANKSSK-amide:

Acetyl-FAKLNC(t-butyl)RLYRKANKSSK-amide was synthesized according to the standard procedure at a 0.1 mmole scale using the duPont RaMPs resin cartridge an cartridge shaker. The peptide was cleaved from the resin and deprotected (except the Cys-S-t-butyl) in several batches using the standard deprotection method. The correct

peptide was purified using isocratic HPLC.conditions (C4-300Å, semi-preparative column, 75/25 H_2O/CH_3CN + 5% TFE/ 0.1% TFA). Amino acid analysis: F 0.78(1,expected) A 2.4(2) K 2.6(4) L 1.6(2) N 2.0(2) C - R 2.5(2) Y 0.96(1) S 2.5(2) FAB-MS: 2124 ($M+H^+$), 2146 ($M+Na^+$) ^1H NMR (D_2O, 300 MHz) d 7.2 (m, 5H, Phe), 6.9 (d, 2H,Tyr 2,6 H), 6.7 (d, 2H, Tyr 3,5H), 4.3 (m), 4.1 (m), 3.8 (m), 3.1-2.5 (m),1.8 (s, 3H, N-acetyl), 1.7-1.1(m), 1.1(s, 9H, Cys -t-butyl), 0.7 (m, 12H, 2 Leu d-CH3).

Acetyl-FAKLNC(S-S-t-butyl)RLYRKANKSSK-amide:

Acetyl-FAKLNC(S-S-t-butyl)RLYRKANKSSK-amide was synthesized according to the standard procedure at a 0.6 mmole scale using the duPont RaMPs resin. Six RaMPs resin cartridges were combined in a glass synthesis vessel. The synthesis was performed employing a wrist action shaker. The peptide was cleaved from the resin and deprotected (except the Cys-S-S-t-butyl) in several batches using the standard deprotection method. The correct peptide was purified using isocratic HPLC.conditions (C4-300Å, semi-preparative column, 74/26 H_2O/CH_3CN + 5% TFE/ 0.1% TFA).

Amino acid analysis: N, 2.1(2 expected) S, 1.8(2) R, 1.9(2) A, 2.0(2) Y, 0.9(1) L, 1.9(2) F 1.0(1) K 3.1(4). FABMS: 2157.1 (M+H)+; MW=2155.2. ^1H NMR (D_2O, 300 MHz) d 7.2 (m, 5H, Phe), 6.9 (d, 2H,Tyr 2,6 H), 6.7 (d, 2H, Tyr 3,5H), 4.3 (m), 4.1 (m), 3.8 (m), 3.1-2.5 (m),1.8 (s, 3H, N-acetyl), 1.7-1.1(m), 1.1(s, 9H, Cys S-S-t-butyl), 0.7 (m, 12H, 2 Leu d-CH3). HPLC: (78% H2O / 22% (95% CH3CN + 5 % TFE)/ 0.1% TFA), 2 ml./ min., Waters C4-300 Å 3.9cm x 30 cm column, elution volume = 24 mls.

ATIII Random:

Acetyl-FKAKNC(t-butyl)RLYRAKSSNLK-amide

Acetyl-FKAKNC(t-butyl)RLYRAKSSNLK-amide was synthesized according to the standard procedure at a 0.1 mmole scale using the duPont RaMPs resin cartridge an cartridge shaker. The peptide was cleaved from the resin and deprotected (except the

Cys-t-butyl) in several batches using the standard deprotection method. The correct peptide was purified using isocratic HPLC.conditions (C_4-300Å, semi-preparative column, 81/19 (H_2O/CH_3CN + 5% TFE/ 0.1% TFA). Amino acid analysis: F 0.35(1,expected) A 2.5(2) K 4.1(4) L 2.0(2) N 2.1(2) C - R 2.2(2) Y 1.1(1) S 2.0(2) FAB-MS: 2125 ($M+H^+$), ^1H NMR (D_2O, 300 MHz) d 7.2 (m, 5H, Phe), 6.9(d,2H,Tyr 2,6 H), 6.7 (d,2H, Tyr 3,5H), 4.3 (m), 4.1 (m), 3.8 (m), 3.1-2.5 (m),1.8 (s, 3H, N-acetyl), 1.7-1.1(m), 1.1(s, 9H, Cys -t-butyl), 0.7 (m, 12H, 2 Leu d-CH3).

Acetyl-FKAKNC(S-S-tBu)RLYRAKSSNLK-amide.

Acetyl-FKAKNC(S-S-t-butyl)RLYRAKSSNLK-amide was synthesized according to the standard procedure at a 0.6 mmole scale using the duPont RaMPs resin. Six RaMPs resin cartridges were combined in a glass synthesis vessel. The synthesis was performed employing a wrist action shaker. The peptide was cleaved from the resin and deprotected (except the Cys-S-S-t-butyl) in several batches using the standard deprotection method. The correct peptide was purified using isocratic HPLC.conditions (Vydac C_4, semi-preparative column, 78/22 H_2O/CH_3CN / 0.1% TFA)

Amino acid analysis: N, 2.0(2 expected) S,1.8(2) R,2.0(2) A, 2.0(2) Y, 0.9(1) L, 1.9(2) F, 0.9(1) K, 2.9 (4). FABMS: 2157.1 $(M+H)^+$; MW=2155.2 ^1H NMR (D_2O, 300 MHz) d 7.2 (m, 5H, Phe), 6.9(d,2H,Tyr 2,6 H), 6.7 (d,2H, Tyr 3,5H), 4.3 (m), 4.1 (m), 3.8 (m), 3.1-2.5 (m),1.8 (s, 3H, N-acetyl), 1.7-1.1(m), 1.1(s, 9H, Cys S-S-t-butyl), 0.7 (m, 12H, 2 Leu d-CH3). HPLC: (79% H2O / 21% (95% CH3CN + 5 % TFE)/ 0.1% TFA), 2 ml./ min., Waters C4-300 Å 3.9cm x 30 cm column, elution volume = 10.4 mls.

ATIII N 135 GlcNAc

Acetyl-FAKLNC(S-S-t-butyl)LYRKAN(GlcNAc)KSSK-amide was synthesized using the standard FMOC stepwise procdure. The peptide was prepared on a 0.15 mmole scale using the Rink Amide MBHA (methylbenzhydrylamine) resin. FMOC-

Asn(GlcNAc)-OH was coupled to the peptide in the same manner as the other amino acids. A two hour coupling produced negative Kaiser test. The following amino acid, Ala, was double coupled. The final amino-acid was added as Acetyl-Phe-OH rather than FMOC-Phe-OH to avoid the acetylation step that is otherwise necessary to "cap" the amino-terminus of the resin. The peptide was cleaved from the resin using the standard procedure. The peptide purified from a broad HPLC peak using isocratic conditions, 75/25 H_2O/CH_3CN/0.1 % TFA on a Vydac C_4 semi-preparative column. Amino acid analysis: Asn 2.1 (2), Ser 1.9 (2) Arg 1.8 (2) Ala 2.0 (2) Tyr 0.9 (1) Leu 1.9 (2) Phe 1.0 (1) Lys 3.9 (4) Plasma Desorption Mass Spec: 2359.8($M+H^+$), 2382 ($M+Na^+$) MW= 2358. Analytical HPLC: 77% H_2O/ 23% CH_3CN/0.1 % TFA, C4-300 A analytical column elution volume = 8 mls at 2 ml./min. ^1H-NMR (D_2O,300 MHz) δ 7.3-7.1 (m), 6.9 (d,Tyr,2,6,H), 6.6 (d.Tyr,3,5 H), 4.9 (d, GlcNAc anomeric proton), 4.5-4.0 (m), 3.8-3.3 (m), 3.1-2.5 (m), 1.85 (s, 3H, peptide N-acetyl CH_3), 1.75 (s, 3H, glucosamine N-acetyl CH_3), 1.7-1.2 (m), 1.1 (s,9H, cys S-S-tert butyl), 0.7 (m, Leu δ-CH_3)

Chapter 3 Appendix A.

^{1}H-NMR Spectra of Protected Fragments

ATIII(123-139) Fragment #1 Boc-FAK(Cl-Z)LNC(4-me-Bzl)R(NO_2)-COOH

ATIII(123-139) Fragment #2 Boc-LY(Cl_2-Bzl)R(NO_2)-COOH

ATIII(123-139) Fragment #3 Boc-K(Cl-Z)ANK(Cl-Z)S(Bzl)S(Bzl)-COOH

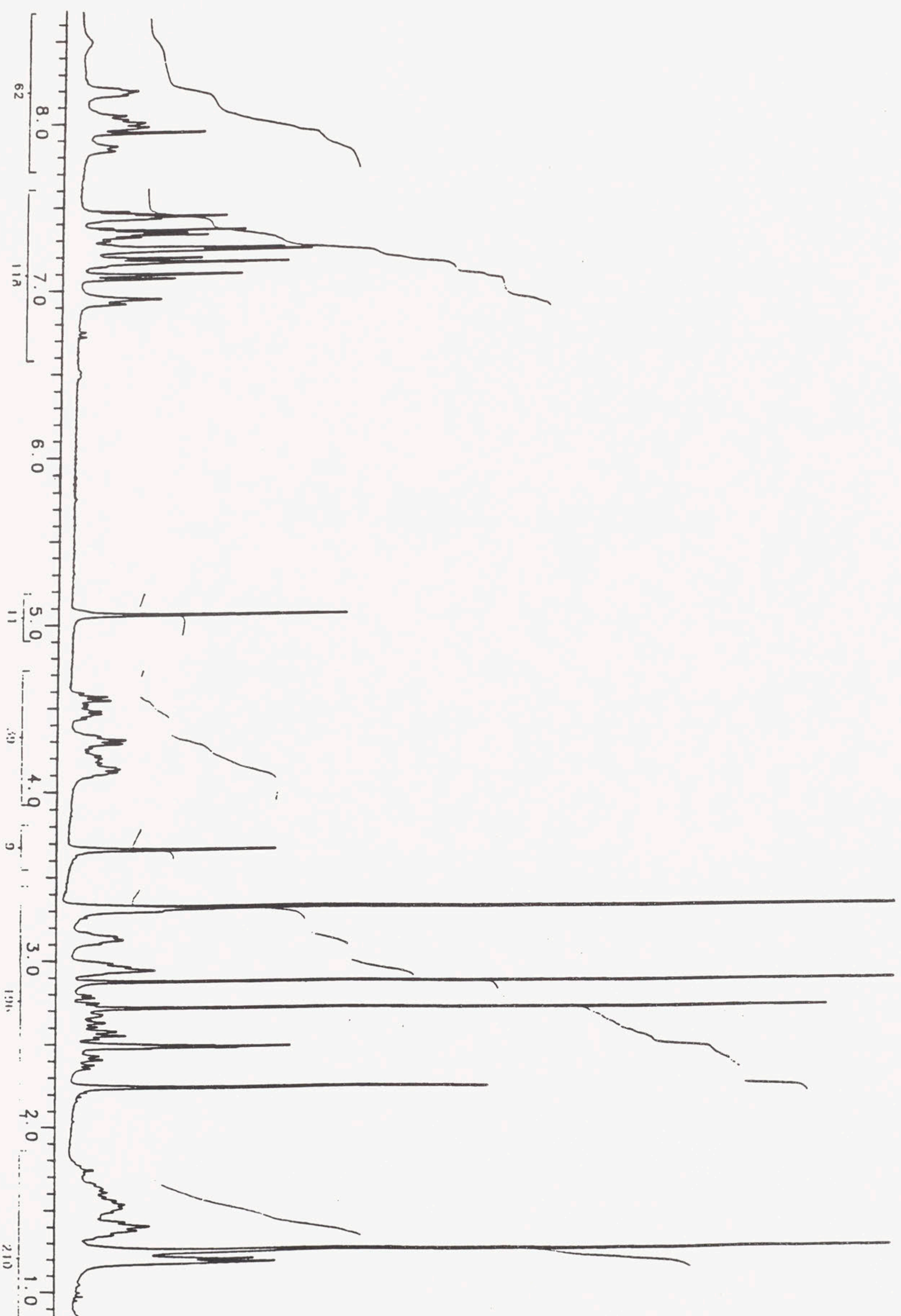
ATIII Random Fragment #1 Boc-FK(Cl-Z)AK(Cl-Z)NC(4-me-Bzl)R(NO_2)-COOH

ATIII Random Fragment #3 Boc-AK(Cl-Z)S(Bzl)S(Bzl)NL-COOH

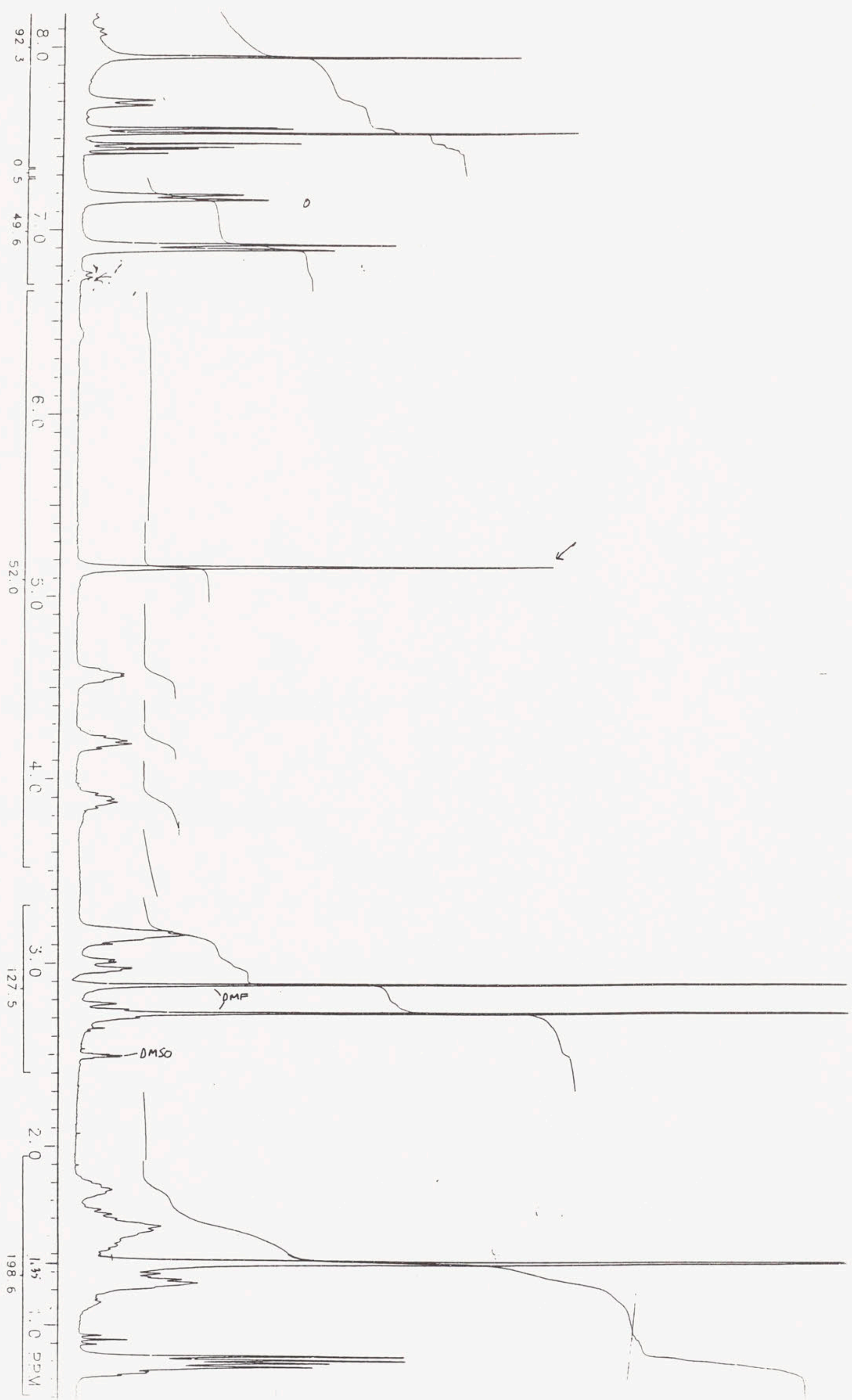
ATIII 2E Fragment #1 Boc-FAK(Cl-Z)LNC(4-me-Bzl)E(Bzl)-COOH

ATIII 2E Fragment #3 Boc-K(Cl-Z)ANE(Bzl)S(Bzl)S(Bzl)-COOH

ATIII(123-139) Fragment #1 Boc-FAK(Cl-Z)LNC(4-me-Bzl)R(NO_2)-COOH

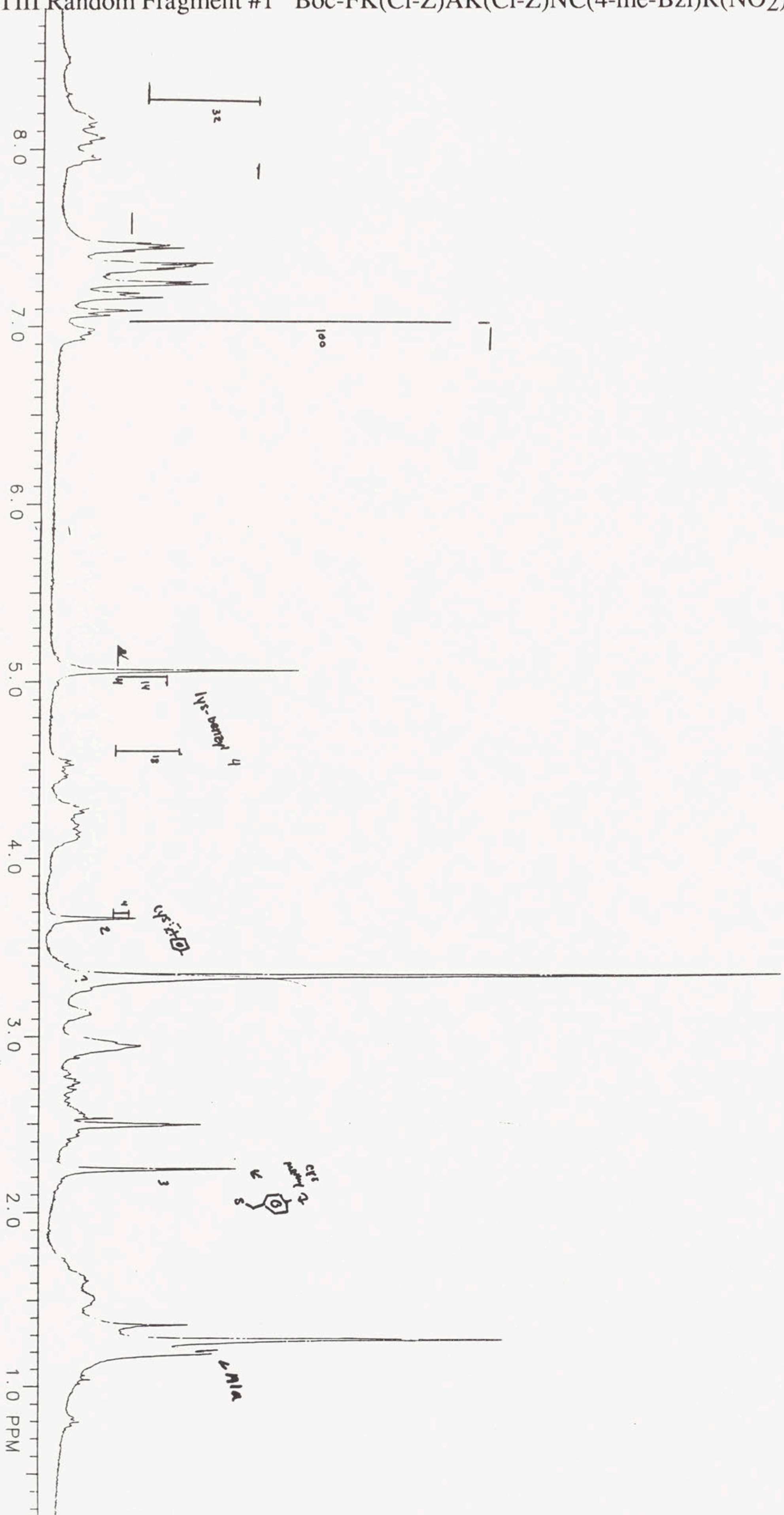


ATIII(123-139) Fragment #2 Boc-LY(Cl_2-Bzl)R(NO_2)-COOH

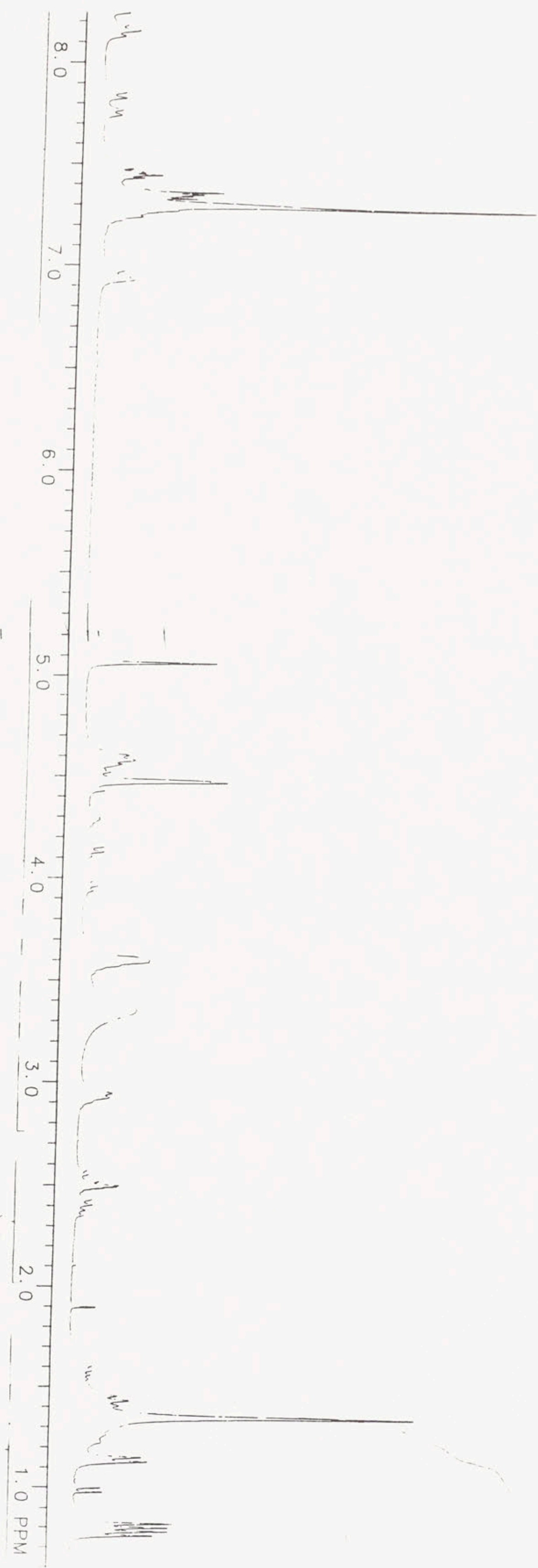


ATIII(123-139) Fragment #3 Boc-K(Cl-Z)ANK(Cl-Z)S(Bzl)S(Bzl)-COOH

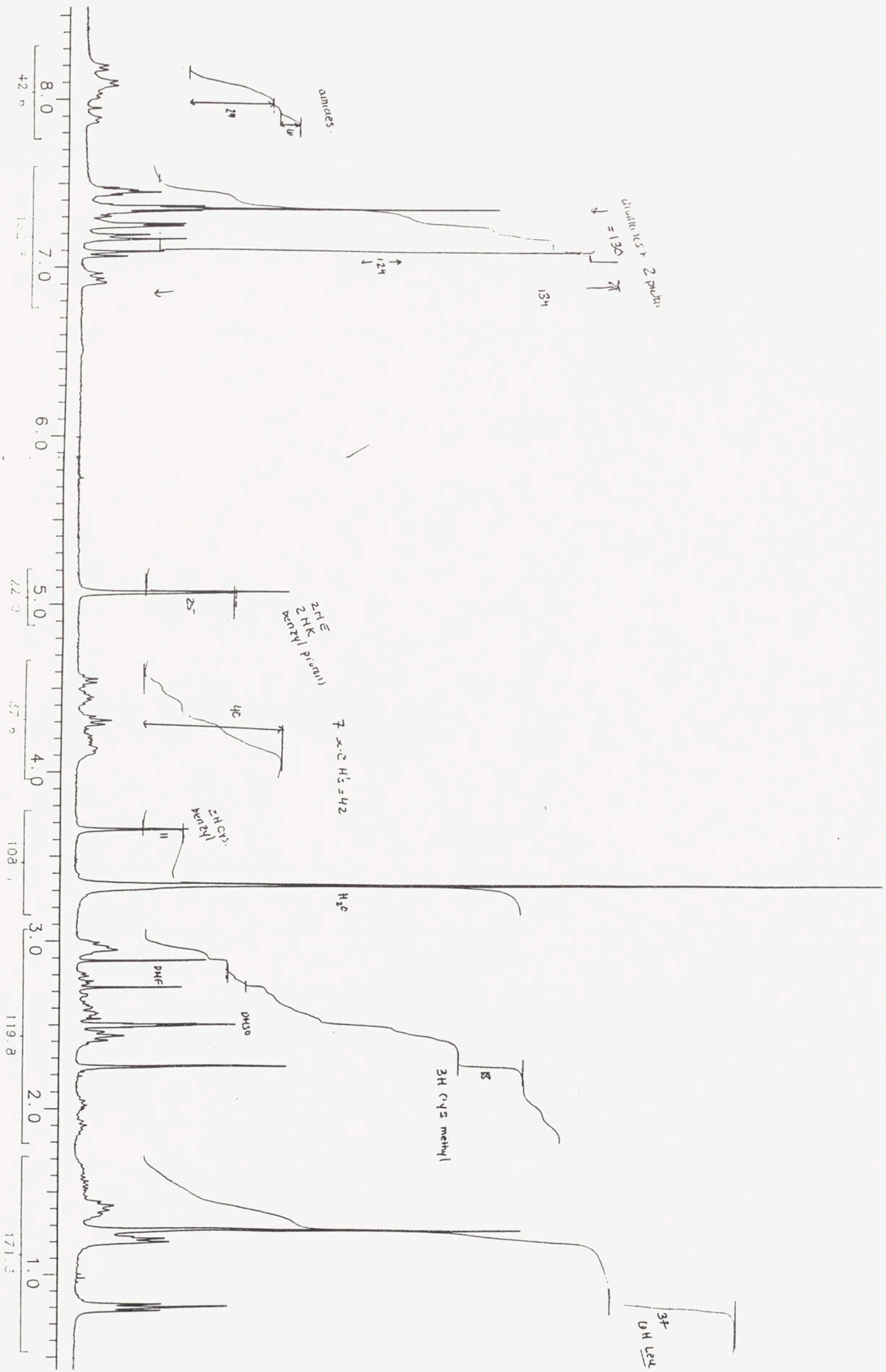
ATIII Random Fragment #1 Boc-FK(Cl-Z)AK(Cl-Z)NC(4-me-Bzl)R(NO_2)-COOH



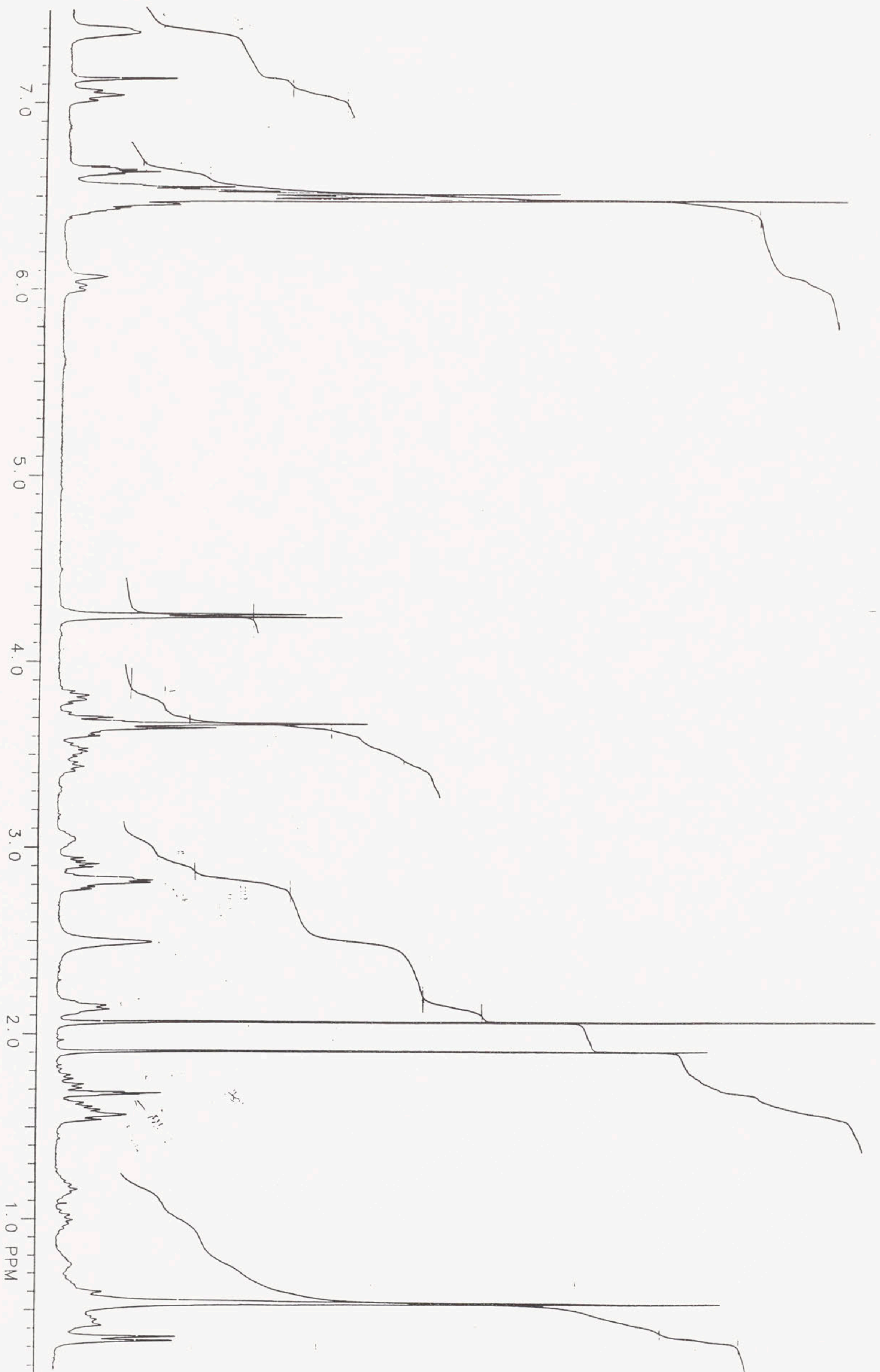
ATIII Random Fragment #3 Boc-AK(Cl-Z)S(Bzl)S(Bzl)NL-COOH



ATIII 2E Fragment #1 Boc-FAK(Cl-Z)LNC(4-me-Bzl)E(Bzl)-COOH



ATIII 2E Fragment #3 Boc-K(Cl-Z)ANE(Bzl)S(Bzl)S(Bzl)-COOH



Chapter 3 Appendix B

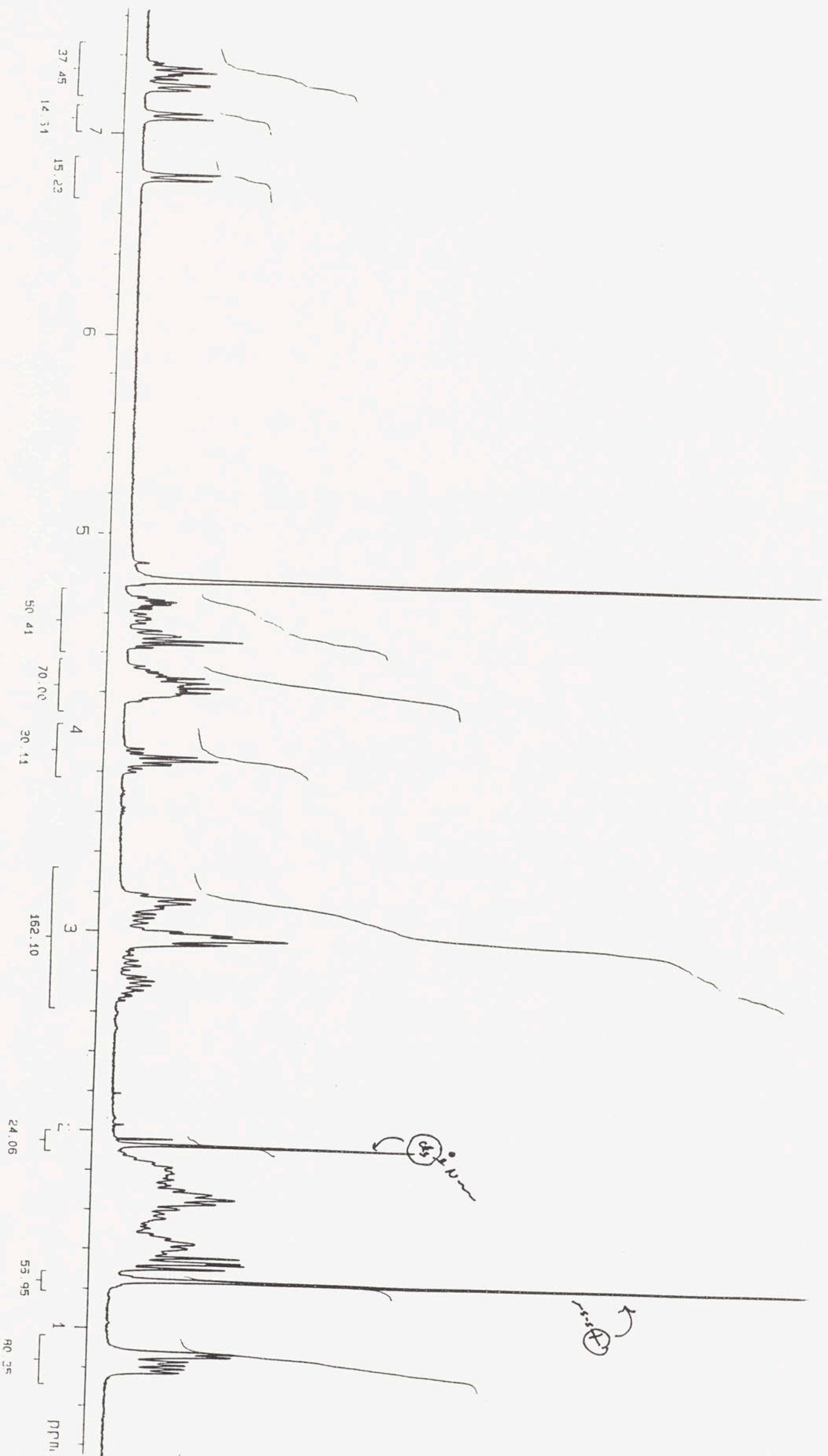
^{1}H-NMR Spectra of Unprotected Peptides

ATIII(123-139) CH_3CO-FAKLNC(S-S-t-butyl)RLYRKANKSSK-$CONH_2$

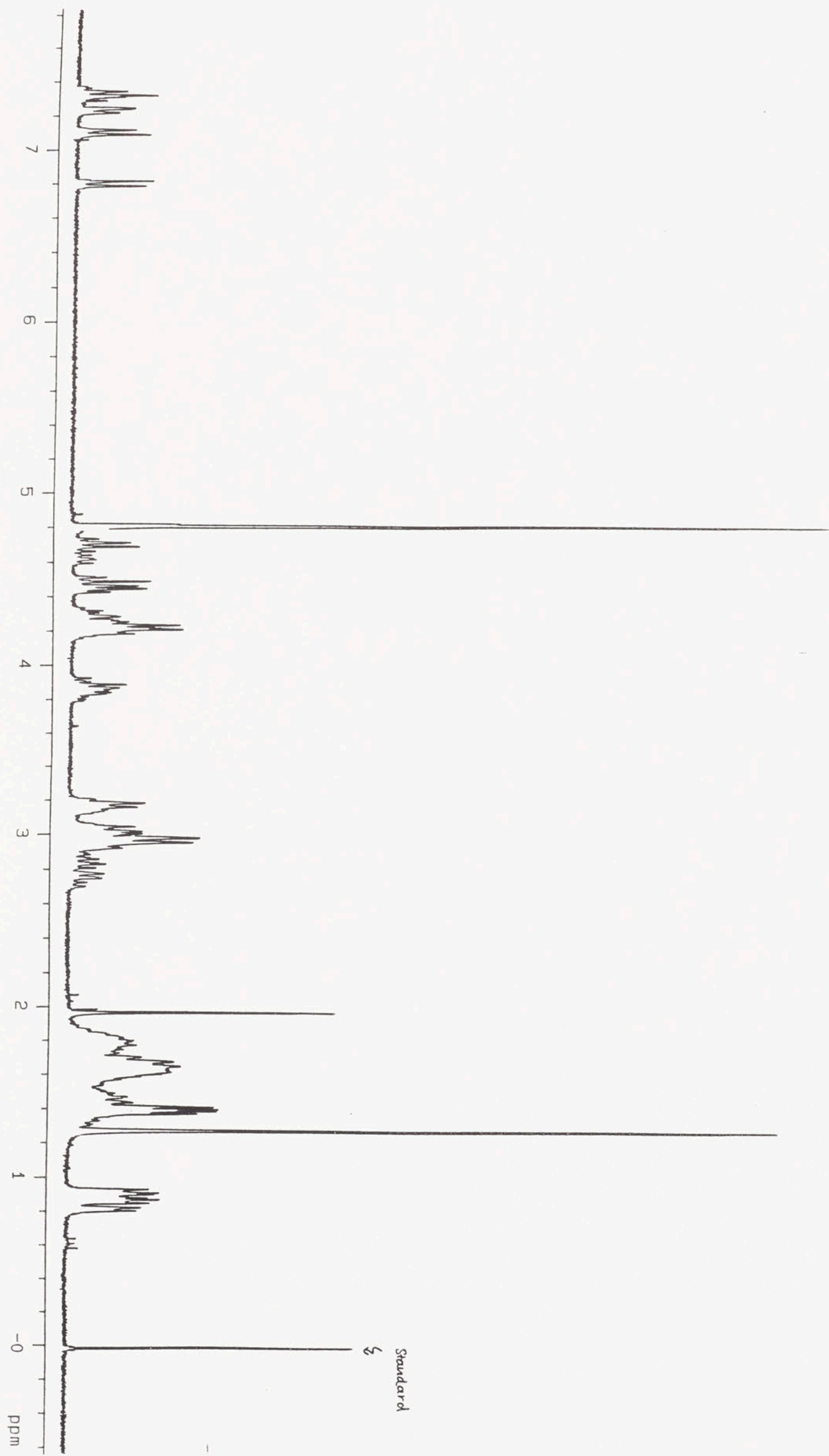
ATIII Random CH_3CO-FKAKNCRLYRAKSSNLK-$CONH_2$

ATIII N135 GlcNAc
CH_3CO-FAKLNC(S-S-t-butyl)RLYRKAN(GlcNAc)KSSK-$CONH_2$

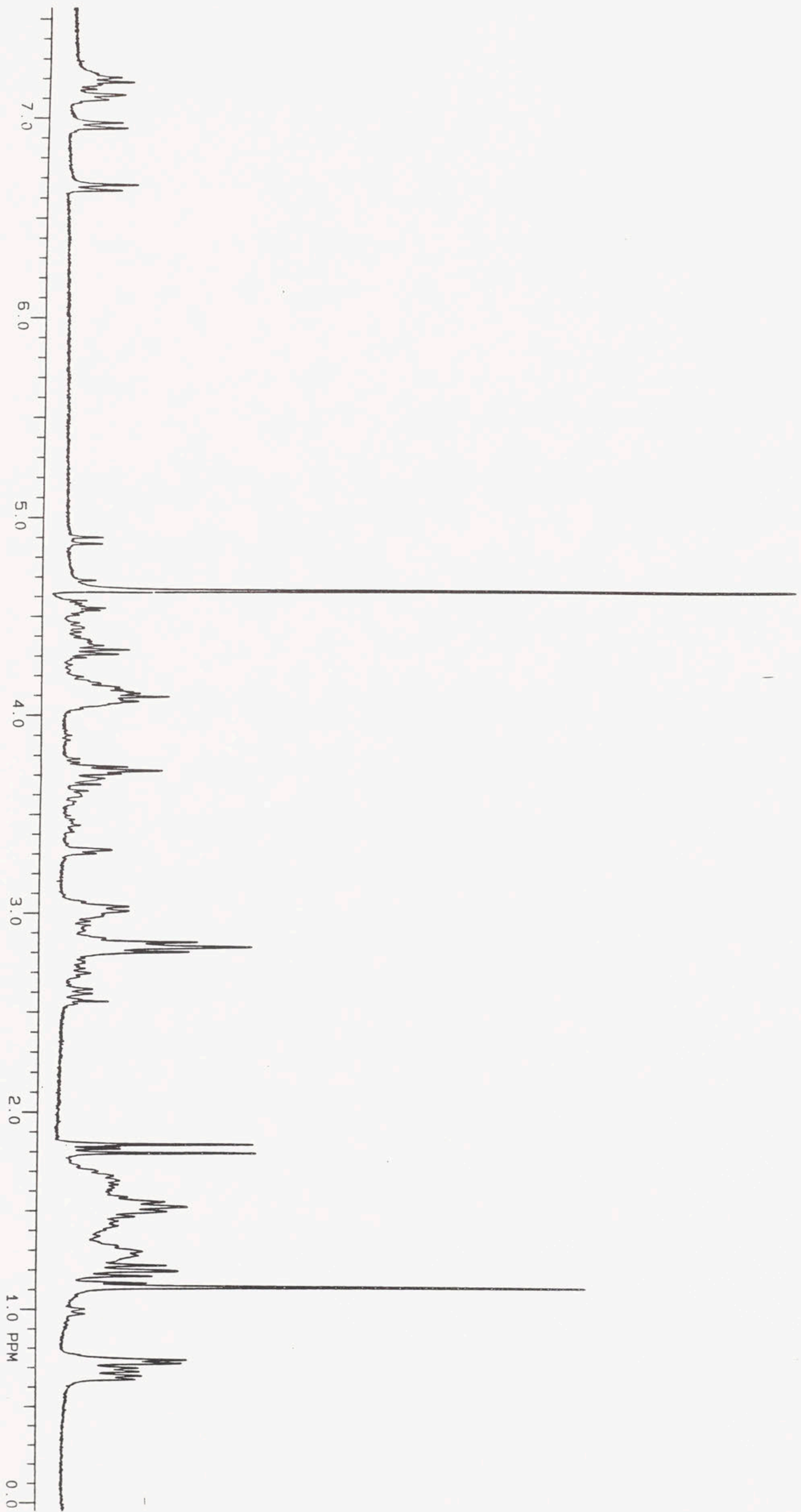
ATIII(123-139) CH_3CO-FAKLNC(S-S-t-butyl)RLYRKANKSSK-$CONH_2$



ATIII Random CH_3CO-FKAKNCRLYRAKSSNLK-$CONH_2$



ATIII N135 GlcNAc CH_3CO-FAKLNC(S-S-t-butyl)RLYRKAN(GlcNAc)KSSK-$CONH_2$



Chapter 4.

The Conformational Properties of the Peptides and the Peptide-Polysaccharide Complexes

Introduction

The peptides ATIII(123-139), ATIII N135 GlcNAc and ATIII Random were prepared as a simple model for the Antithrombin III-heparin interaction. The conformational behavior of these peptides in the presence and absence of heparin is a central part of this model It has been hypothesized that heparin binding stabilizes α-helical structure in ATIII. Circular dichroism (CD) spectroscopy is particularly useful for detecting α-helical peptide structures. and was therefore used to assess the conformational behavior of the peptides and the peptide-polysaccharide complexes.[1] Where possible, Fourier transform infrared (FTIR) spectroscopy was also applied. Both CD and FTIR have been used extensively in the study of peptide and protein secondary structure.

This chapter will first discuss the application of CD to the study of peptide and polysaccharide conformation. The conformational propensities of ATIII(123-139), ATIII Random and ATIIIN135 GlcNAc will be presented and discussed. Finally, the

conformational properties of each of the peptides in the presence of heparin and two other sulfated polysaccharides will be presented and discussed.

Circular Dichroism Spectroscopy as a Tool for Probing Peptide Conformation

Circular dichroism (CD) spectroscopy has grown in popularity to become a standard part of the experimental repertoire of many protein chemists and structural biologists. CD spectroscopy is a variation of UV-VIS (electronic absorption) spectroscopy that uses chirally polarized light rather than normal isotropic light as its source. [2] In a CD spectrometer, light from a powerful source is modulated to generate a beam comprised of right- and left- circularly polarized components. A chiral molecule (or UV active functionality in a chiral environment) will absorb different amounts of each circular component of the light . The instrument detects the difference in absorption of each of the two circular components (ΔA). For historical reasons, most CD spectrometers report the signal they measure in terms of ellipticity (θ) with a unit of degrees.[3] The use of ellipticity units refers to the fact that linearly polarized light becomes elliptically polarized when absorbed by an asymmetric sample. The difference in absorbence is related to ellipticity by $\Delta A= \theta/32.98$. [4]

The CD signal of an optically active compound occurs generally in the region of the spectrum where it would normally absorb in standard UV-visible spectroscopy. The CD signal or curve in that region is called a Cotton effect curve and characteristically has either a positive or negative signal. In proteins and peptides, the amide bonds , disulfide bonds and aromatic side chains are all CD active.[3] The amide bond CD signal, which occurs in the far UV (250 nm -185 nm), is most commonly studied because it is extremely sensitive to changes in secondary structure of the polypeptide chain and therefore most informative. In UV-visible spectroscopy, the amide bond is characterized by a weak but broad n to π^* transition at 210 nm and an intense π to π^* transition at 190 nm.[4] The backbone amide bond is active in CD spectroscopy because it is situated in between two chiral centers (amino acid α-carbons) and is therefore in a disymmetric

environment..[5] In the CD spectrum, signals corresponding to each of the electronic transitions of the amide bond are observed. The wavelength, magnitude, and sign (+/-) of the amide CD signal is diagnostic of the presence of secondary structure. Each type of secondary structure produces a characteristic spectrum. Figure 1 shows spectra of each of four major categories of secondary structure, α–helix, β-sheet, β-turn and random coil. Two sets of spectra are shown, whose source will be discussed later in this section.

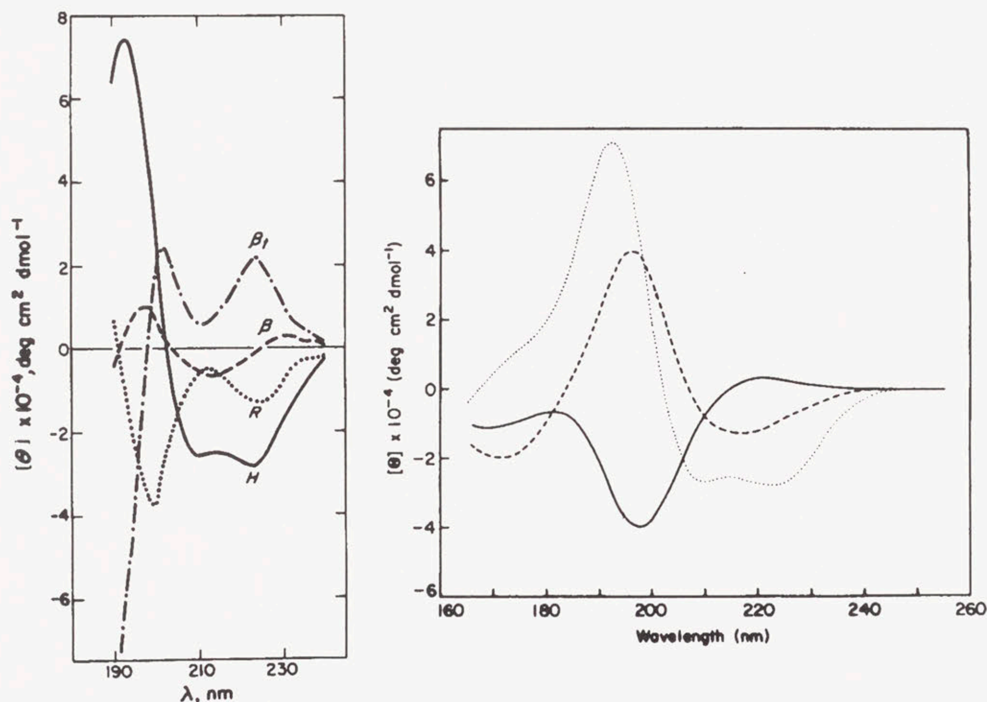


Figure 1. Reference Spectra for Secondary Structure
Left: Spectra derived from CD of proteins of known structure[6]
Right: Spectra of peptidyl homopolymers of a unique structure[7]
(solid line:random, dots:α-helix, dashes: β-sheet)

Disulfide bonds, as well as the aromatic side chains of tyrosine, phenylalanine, and tryptophan, all have CD activity in the near UV (260-300) nm. The magnitude of these signals is lower (~ 1/10th) than that of the backbone amide bond.[8] Although changes in these signals are observed for proteins in different environments, it is not clear how these changes correlate with changes in secondary structural.

The interpretation of protein and peptide CD spectra is based on the idea that the each type of secondary structure (helix, sheet, turn, or random) has a characteristic CD signal which is combined linearly in the sample spectrum. Therefore, a sample CD spectrum can be decomposed into contributions from each type of secondary structure. A large number of computer programs have been written to facilitate CD spectral interpretation.[6, 7, 9, 10, 11] These fitting programs use of a set of reference spectra represent ing the pure secondary structural motifs. See Figure 1. Most programs emoploy some type of repetitive least squares analysis (single value decomposition) to generate a theoretical spectrum from the reference set which approximates the observed spectrum.[3] The fractional composition of secondary structure in the theoretical spectrum is then an approximation of the secondary structural composition of the observed spectrum.

There are several potential sources of inaccuracy in the interpretation of CD spectra; the spectra to beinterpreted, the source of the reference set used in a fitting program.and the method by which a "good fit" for the observed spectrum is chosen. In a recent review by Johnson[1],. it is stressed that in order to accurately decompose a spectrum into its structural components, the informational content of the spectrum must be as high as possible. With the exception of assigning α-helical structure (contributing signals at 222 nm.), there is insufficient information to accurately decompose the spectrum unless the data extends to 184 nm.

Fitting program reference sets are taken from either the CD spectra of peptidyl homopolymers that are assumed to be of purely one type of secondary structure (as in Figure 1, right)[7] or from a compilation of CD spectra of proteins whose structures have been determined by X-ray crystallography (as in Figure 1, left).[6] By comparing the reference spectra shown in figure , it is obvious that the spectra assigned to the specific structural motifs differ significantly between the two sources.

The use of peptidyl homopolymers as a reference has been criticized because the structures in the long homogeneous peptide chains are not thought to be representative of

the structures found in a protein. For example, an α-helical signal is known to be chain length dependent [12]. The helices in a protein are generally short, only 10-12 residues long. The helices in a homopolymer are effectively infinitely long.[12] Homopolymers provide limited contributions from side chains,[13] while there are few good examples of pure β-turn structures.[9] Reference spectra derived from proteins are therefore deemed more relevant for the study of protein structure. However, the reference spectra are inherently biased towards that set of proteins from which the reference set was created.

The criteria used to judge the "goodness" of fit to the observed spectrum is another potential source of inaccuracy. A fitting program calculates a theoretical spectrum and analyzes the difference between the theoretical spectrum and the observed spectrum. How the program chooses to weight the difference and proceed with the calculation will effect the final fit. When the CD spectra of proteins of known structure are used as a reference, the number of spectra is usually greater than the informational content of the data to be analyzed. Therefore, a large number of solutions will fit the data within the limits of error defined by the program.[3]

In this project, CD spectroscopy was employed primarily for its ability to detect changes in peptide conformation. Previous studies have shown that CD is useful in detecting structural changes in peptides in peptide-ligand interactions. α–Helical structures , unlike β-sheet structures, are particularly easy to detect. It was hoped that CD could be used to define a peptide-polysaccharide complex with secondary structure that could be studied further using NMR spectoscopy.

<u>The Structural Properties of ATIII(123-139), ATIII Random and ATIII N135(GlcNAc) as Determined by CD spectroscopy</u>

Before examining the peptides in the presence of the polysaccharides, it was important to determine the conformational properties of the peptides alone. The peptides were therefore studied under a variety of potentially conformation altering conditions as summarized in the following pages.

In all the experiments described in this chapter (as well as chapter 5), the peptide concentrations were determined using the UV absorbence of tyrosine for peptide samples in 6 M Gdn Hcl. Quantitative amino acid analysis of peptide stock solutions gave widely variable results that were consistently low compared to the very simple and direct tyrosine absorbence concentration values. In theory, quantitation by tyrosine absorption is very accurate [14]provided the peptide sample is pure.

The peptides were found to have no stable secondary structure in aqueous solution.

Initially, the spectra of each of the peptides was determined in a neutral aqueous buffer. After trying many different conditions, a 50 uM peptide solution in a 1mm pathlength cell was found to be optimal for this series of peptides. Mono sodium phosphate and tris were found to be suitable buffering agents. Although NaCl was used in most experiments, KF later proved to have less absorbance below 200 nm. The CD spectra were obtained from 250 to 195 nm.

The spectra of each of the three peptides exhibited the intense minimum at 200 nm characteristic of peptides in a random ensemble of conformations.[3] See figures 1A,B, and C. This finding was consistent with the FTIR spectra in D_2O where the amide I stretch of each peptide was centered around 1650 cm^{-1}, again indicative of a random ensemble of conformations.[215]

Inspection of the peptide sequences reveals that while all the charged residues segregate to one face of the helix, the non-polar groups segregate to another. This suggests that at some critical concentration the peptide might form multimeric structures, such as amphipathic helical dimers. Peptides samples ranging in concentration from 5uM-200 uM were examined. The CD signal increased linearly with the peptide concentration indicating that no cooperative structure formation was taking place (at least at the concentrations the CD experiments were to be performed).

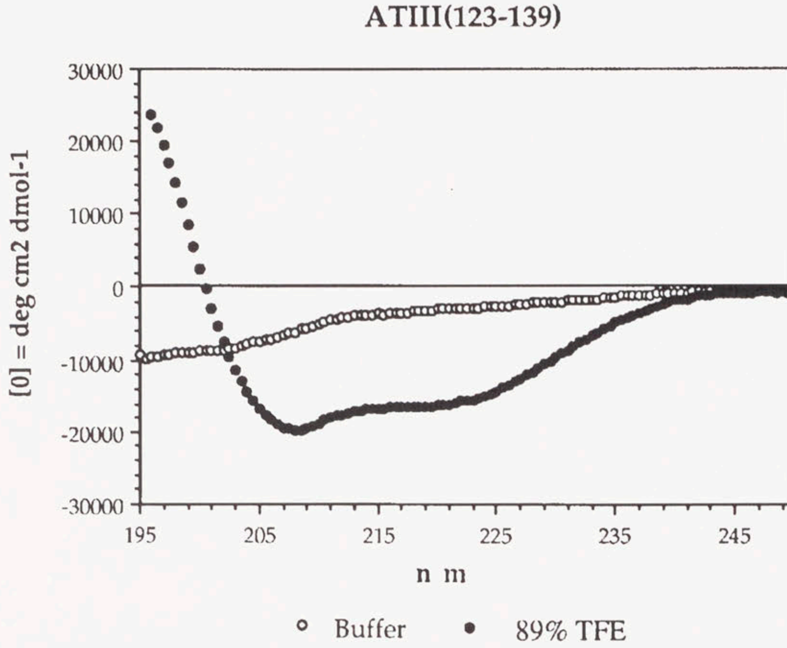


Figure 2 A. 50 uM ATIII(123-139) in 10 mM phosphate, 50 mM KF, pH 7 or TFE

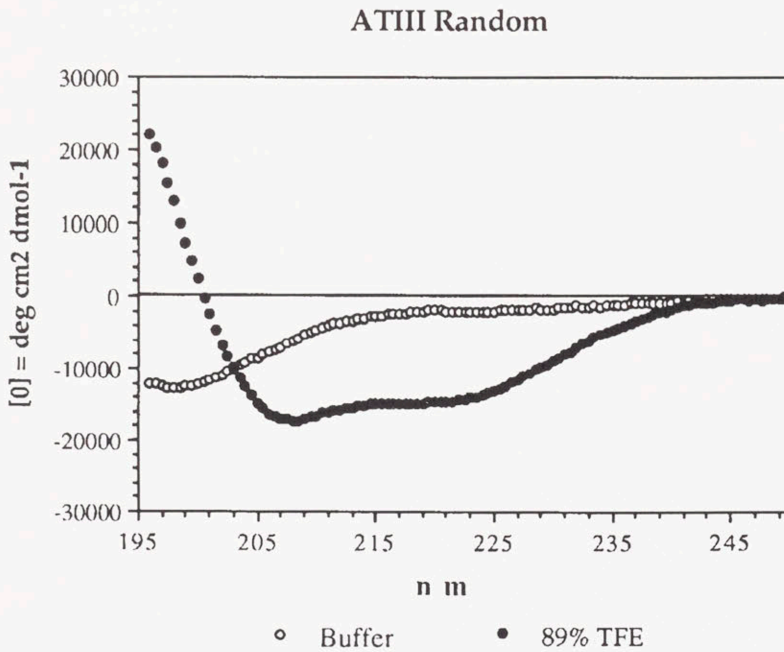


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Figure 2 B. 50 uM ATIII Random in 10 mM phosphate, 50 mM KF, pH 7 or TFE

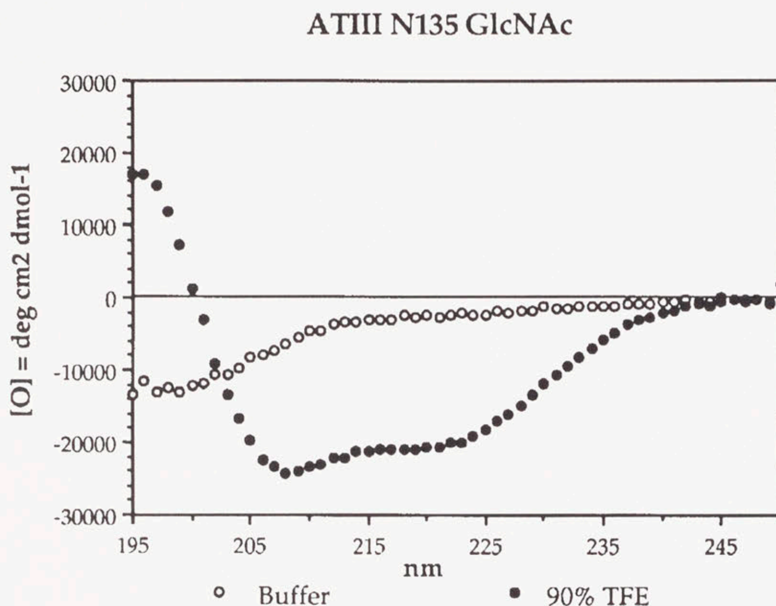


Figure 2 C. 50 uM ATIII N135 GlcNAc in 10 mM phosphate, 50 mM KF, pH 7 or TFE

The Peptides were Insensitive to Changes in pH or Ionic Strength of Their Environments

Charge neutralization had been hypothesized to play an important role in the Antithrombin III-heparin interaction as potential driving force for stabilization of secondary structure. Each of the peptides prepared contained six positively charged side chains within a 17 residue chain. It was therefore important to determine whether the peptides were sensitive to changes in the ionization state of the amino acid side chains, or the ionic strength of the solvent.

The effect of pH on the peptides was determined by obtaining the CD spectra in solutions of pH 1, 6, and pH 12. At pH 1 the ionization state of the peptides should be unchanged.from that of neutrality At pH 12, the four lysine ε-amines should be 99% deprotonated (pKa ~ 10) and the arginine guanidinium groups at least partially deprotonated (pKa~ 12.5). Homopolymers of polylysine and polyarginine are classically known to assume α-helical structure at high pH.[16] It was thought that at high pH, either ATIII(123-139) or ATIII Random might also assume α-helical structure. The CD spectra of the ATIII(123-139) at each pH were superimposable. (Figure 3) The spectrum

of ATIII Random at pH 12 was different from those at pH 1 and 6, but not in any interpretable fashion. (Figure 4)

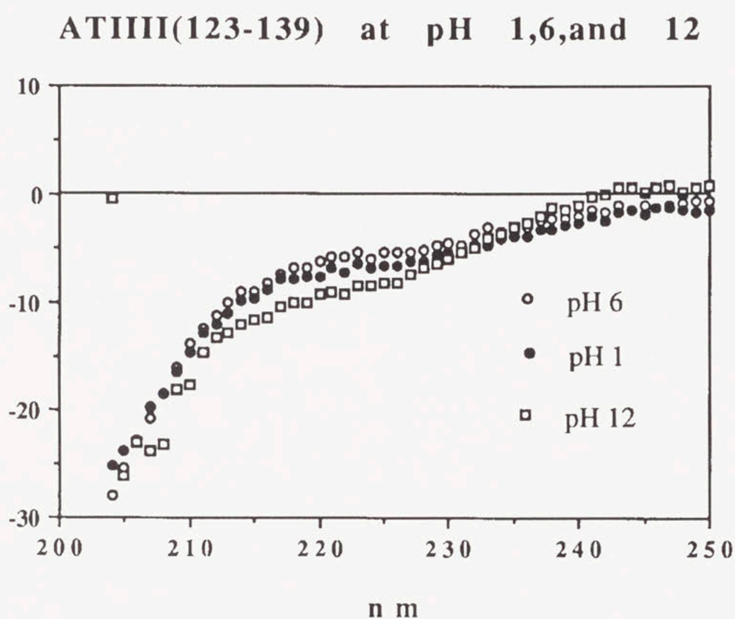


Figure 3. 75 uM ATIII(123-139) in solvents of various pH.

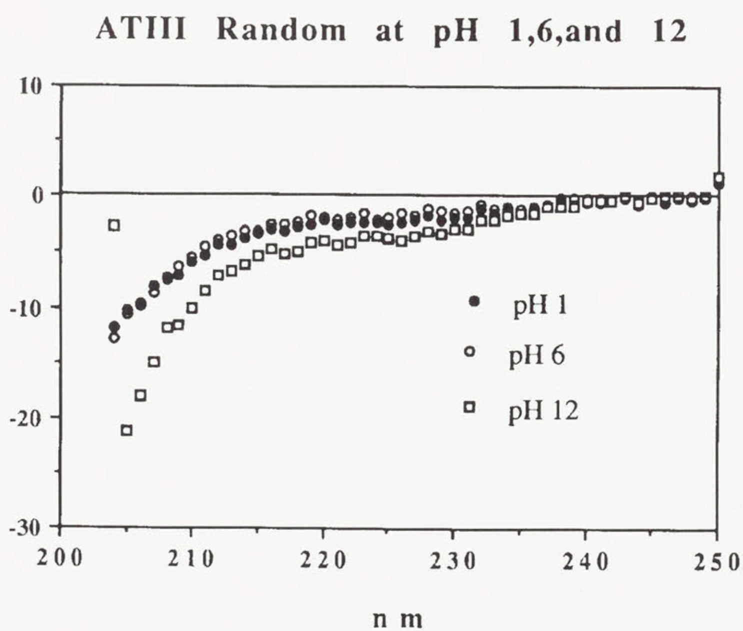


Figure 4. ATIII Random in solvents of various pHs.

The effect of changes in the ionic strength of the solvent was also explored. As the ionic strength (dielectric constrant) of a solvent increases, the electrostatic attractions and repulsions between ions is reduced. Peptide conformations otherwise unfavorable because of electrostatic repulsions between side chains may be stabilized at high ionic strength. For example, high salt has been shown to induce β-sheet structure in homopolymers of poly(lys-phe).[17] CD spectra of each of the peptides was obtained in 1 M NaCl, Na SO_4 and $LiClO_4$, however the spectra were indistinguishable from those of the peptides in buffer alone.

In the presence of an organic solvent, α-helical structure was stabilized.

The induction of or stabilization of helical structure is a central issue in the discussion of the heparin-Antithrombin III interaction. The sequence synthesized, 123-139, had been proposed to be helical in the native protein.[18] It was therefore important to demonstrate that the peptide sequences we chose were indeed capable of forming helices under the appropriate conditions.

Organic solvents have long been known to induce helical structure in peptides. By lowering the polarity of the solvent, intramolecular hydrogen bonding (such as NH_i to CO_{i+4} hydrogen bonding found in α-helices) is favored over hydrogen-bonding from the peptide to the solvent. The behavior of a peptide in TFE can be taken as an indication of the helix forming propensity of the peptide.[19] Following the growth of structure in a peptide as a function of TFE concentration is analogous to following the denaturation of a globular protein in urea. Both procedures are indirect measures of the stability of secondary (tertiary)structure.

The CD spectra of all three peptides in TFE showed the double minima at 208 and 222 nms characteristic of helix formation. See Figures 2 A, B, and C.

	% Heliciry
ATIII(123-139)	46%
ATIII Random	48%
ATIII N135 GlcNAc	58%

The 222 nm signal is unique to helices. The other secondary structures have minimal contributions at this wavelength. The helical content reported above for each of the peptides in TFE was derived from the following equation.[20, 21]

$$\% \ \alpha\text{-helix} = ([\theta] + 3000/39000) \times 100$$

where [θ] is the mean residue ellipticity at 222 nm.

ATIII(123-139) was expected to assume less α-helical structure in TFE than ATIII Random. Refering again to the helical wheel diagram in Ch. 2 (Figure 2, p. 36), the charged residues of ATIII(123-139) would segregate to one face of the helix while they would be equeally distributed in the ATIII Random sequence. Lowering the polarity of the solvent is expected to enhance the electrostatic repulsions between the charged side chains, thereby disfavoring the formation of structures (helical ATIII(123-139)) in which like charges are brought together.

Another agent found to induce α-helical structure in both ATIII(123-139) and ATIII Random (ATIII GlcNAc wasn't tested) was the anionic detergent sodium dodecyl sulfate (SDS). At 10 mM, 2mM SDS molecules are in micelles, while the rest are free in solution. [22] A micelle presenting a surface of sulfate ions could be similar to the array of sulfate ions thought to be presented by heparin. No further investigation of this finding was made.

ATIII(123-139) and ATIII N135 GlcNAc have Similar Conformational Behavior.

ATIII(123-139) and ATIII N135 GlcNAc differ only by the presence of the N-acetyl glucosamine residue on Asn135. In buffer and TFE, their behavior is very similar. The influence of covalently linked glycosyl moieties on peptide or protein conformation

has not been clearly defined. However, Otvos et al. report that N-linked disaccharides (chitobiose) change the structure forming capacity of some peptides.[23]

CD Spectroscopy of Sulfated Polysaccharides

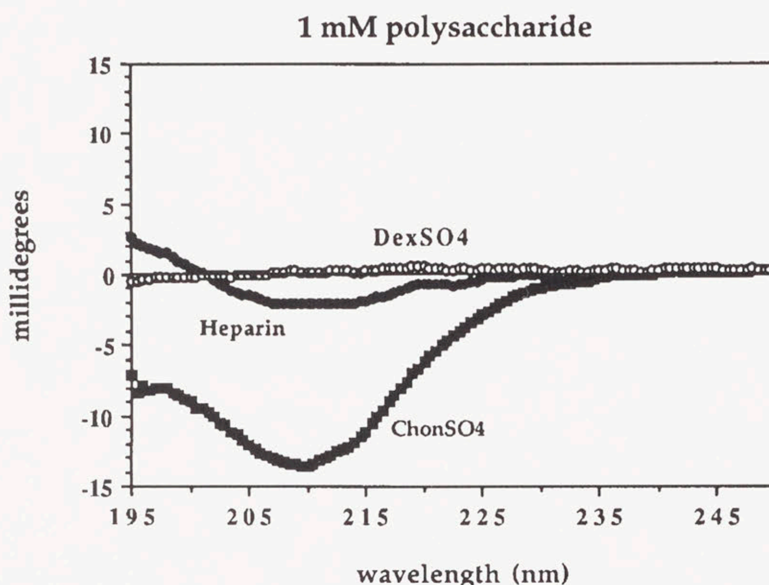


Figure 5. CD Spectra of 3 Sulfated Polysaccharides

CD spectroscopy is not commonly used in the study of glycosamiminoglycans chain conformation. The molar contributions from the saccharides are relatively weak. It is not known how changes in the CD spectra correlate to changes in the chain conformation. The spectra shown in figure 5 are reported in millidegrees unlike peptide spectra whose signals areexpressed as millidegree/M peptide(see experimental section)

The CD signals exhibited by glycosaminoglycans are attributable to the amide functionalities of the amino sugar residues as well as the carboxylates of the uronic acid residues. All glycosaminoglycans exhibit a negative cotton effect in the region of 208 to 212 nm corresponding to the n-π* transitions of the amide. The carboxylate n-π* transition is observed in this region too. See reference #[24] and citations therein. Differences in the Cotton effect of the amide π-π* transition divides the glycosaminoglycans into two classes. Heparin, keratan sulfate and heparan sulfate have a positive Cotton effect at 188 nm. Chondroitin 6-sulfate, chondroitin 4-sulfate and

hyaluronic acid have a more intense negative Cotton effect at 185 nm. The difference in sign of the π–π* transitions of the two classes has been proposed to result from difference in substitution at the 3 position of glucosamine. The chondroitin sulfates have a glycosidic linkage at the 3-position whereas members of the heparin class have a hydroxyl group. The functionality at the 3 position may alter the orientation of the N-acetyl group on the 2 position.

Chondroitin 6-sulfate, a glycosaminoglycan, and dextran sulfate, a sulfated polysaccharide, were used as heparin controls in experiments described in this chapter and chapter 5. Their structures are shown below.

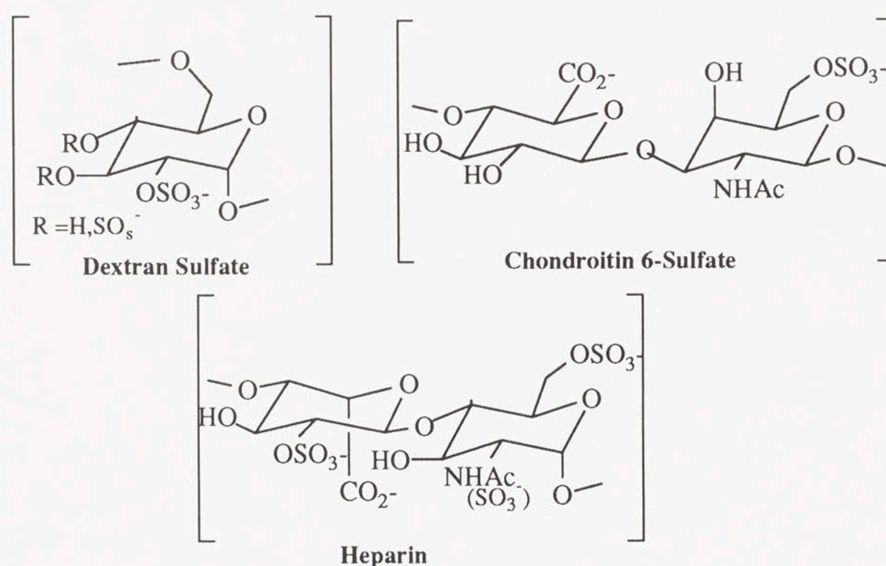


Figure 6. Disaccharide units of heparin, chondroitin 6-sulfate and dextran sulfate

The Structure of the Peptide-Polysaccharide Complexes

Heparin was found to induce structure in ATIII(123-139) but not ATIII Random

The peptides ATIII(123-139) and ATIII Random were first studied in the presence of heparin. ATIII(123-139) was found to undergo a dramatic conformational change upon addition of heparin while ATIII Random did not. Furthermore, the CD of the ATIII(123-139)-heparin complex showed none of the characteristics of an a-helix (double minima at 208 and 222 nm) but rather ressembled that of a β-strand or β-sheet.

with a single minimum at 217 nms and a crossover point at 205 nms.[25] Figures 7A and 7B show the spectra of each of the peptide-heparin complexes as compared to the sum of the spectra of peptide and heparin alone. Because heparin has a CD signal in the same region as the peptide, it would not be correct to compare the spectrum of the complex to that of the peptide.

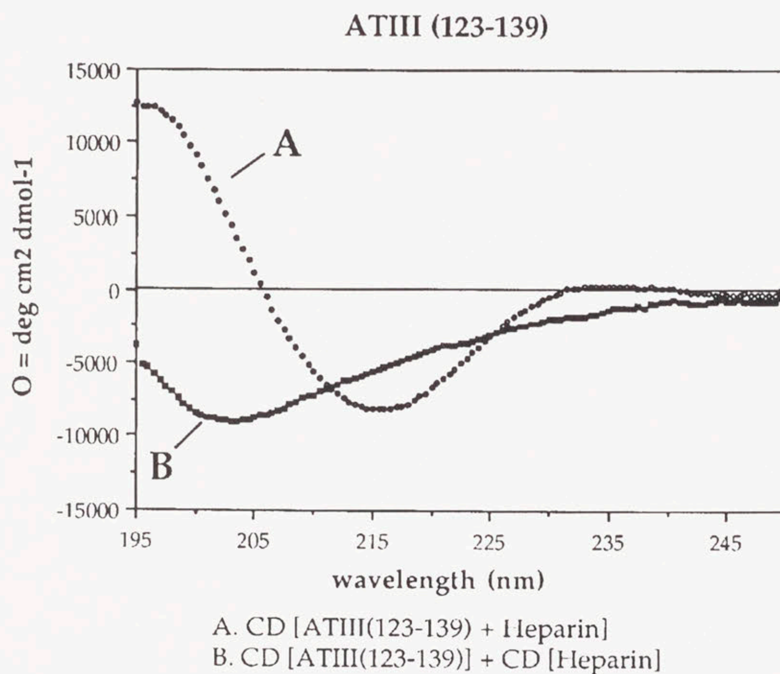


Figure 7A

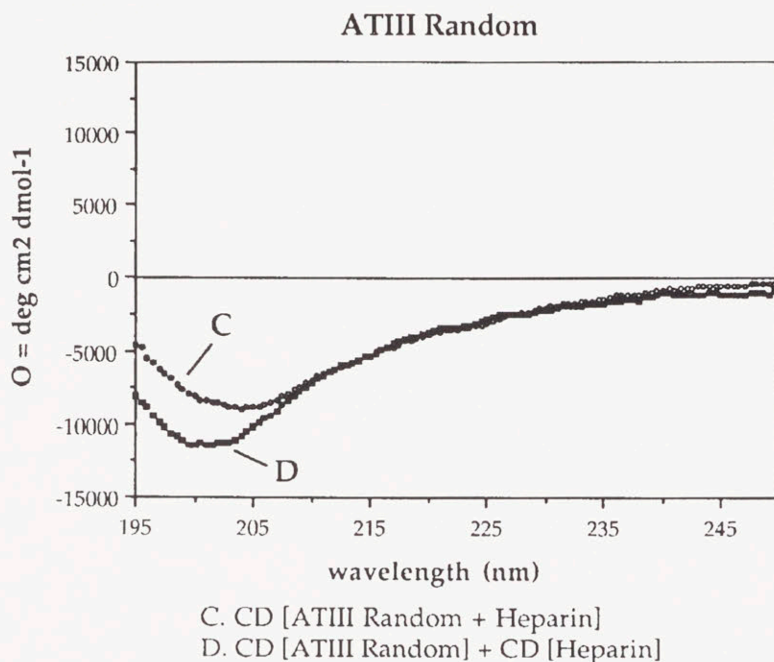


Figure 7 B

Heparin- ATIII(123-139) vs.Heparin- ATIII N135 GlcNAc

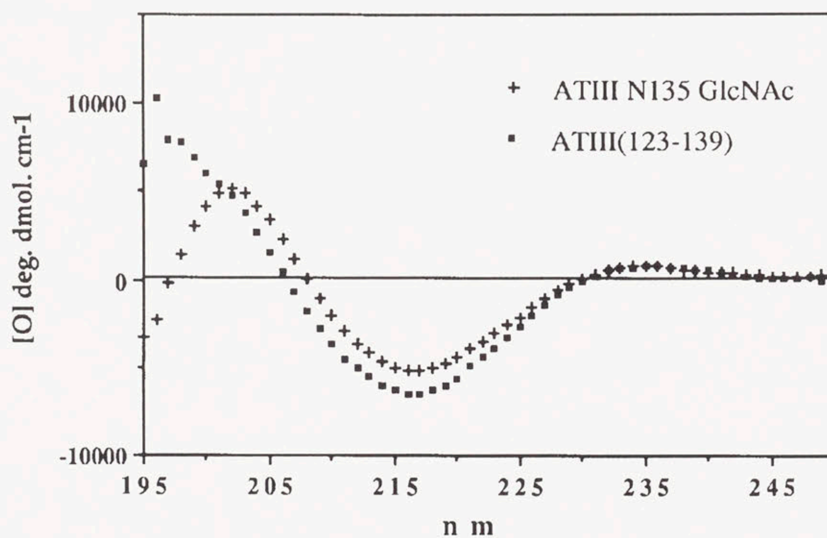


Figure 8..

As shown in Figure 8., the glycopeptide ATIII N135 GlcNAc also assumes structure in the presence of heparin. The minimum at 217 is similar, but the maximum peak wavelength has shifted significantly. A positive peak above 200 nm may be indicative of β-turn structure.[3]

In later experiments where the peptide-heparin complexes are compared to complexes with other polysaccharides, the polysaccharide contribution is subtracted from the complex spectrum. This practice raises the point that in any of the peptide-polysaccharide complex CD spectra, we assume that changes observed are due to changes in the spectral contributions of the peptide and not the polysaccharide. We found no reports in the literature citing examples of large changes in CD spectra of glycosaminoglycans resulting from ligand binding. However, because the peptide and glycosaminoglycan CD signals occur at overlapping wavelengths, we cannot prove conclusively that changes observed upon complexation are strictly the result of changes in the peptide conformation.

One effort made to validate our assumption was to deacetylate heparin and show that the change had negligible effect on the CD spectrum of the complex. The N-acetyl amide groups of the heparin glucosamine residues contribute to heparin's CD signal at 210 nm.. Deacetylation of heparin has been shown to have negligible effect on the anit-coagulant activity of heparin.[26] Deacetylated heparin has a smaller CD signal than crude heparin (350 deg cm^2 $dmol^{-1}$ vs. 1250 deg cm^2 $dmol^{-1}$) The spectra of ATIII(123-139) with heparin and deacetylated heparin, each corrected for their respective heparins, were virtually superimposable. (See Figure 9).

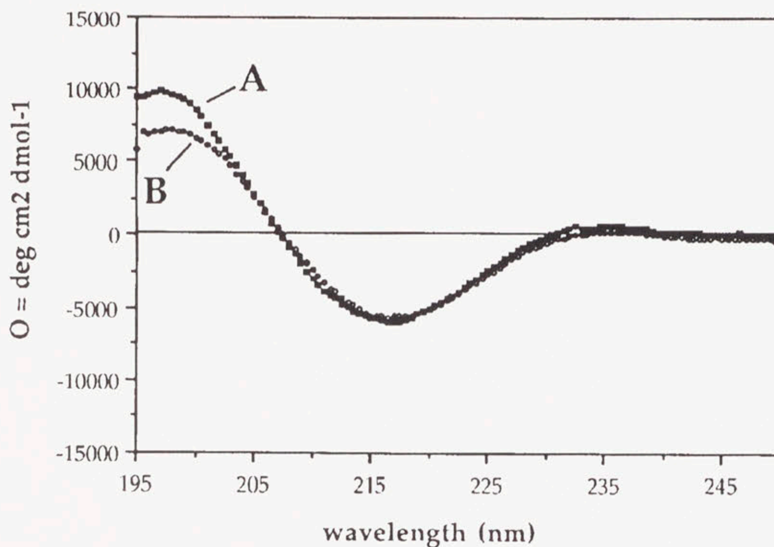


Figure 9 A. CD[ATIII(123-139) + heparin] - CD[heparin]
B. CD[ATIII(123-139) + deacetylaed heparin] -CD[deacetylated heparin].

Other Polysaccharides were capable of inducing structure in ATIII(123-139) but not ATIII Random.

In order to test if structure could be induced in the peptides by other sulfated polysaccharides, chondroitin 6-sulfate and dextran sulfate were tried. These two polysaccharides have negligible anti-coagulant activity. The same trend was observed. ATIII(123-139) assumed structure in the presence of the polysaccharide while ATIII Random did not. The spectra of ATIII(123-139) with each of the three polysaccharides are similar in that they show no obvious characteristics of an a-helix, but they vary in the magnitude and wavelength of their single minima. (See Figure 10.)

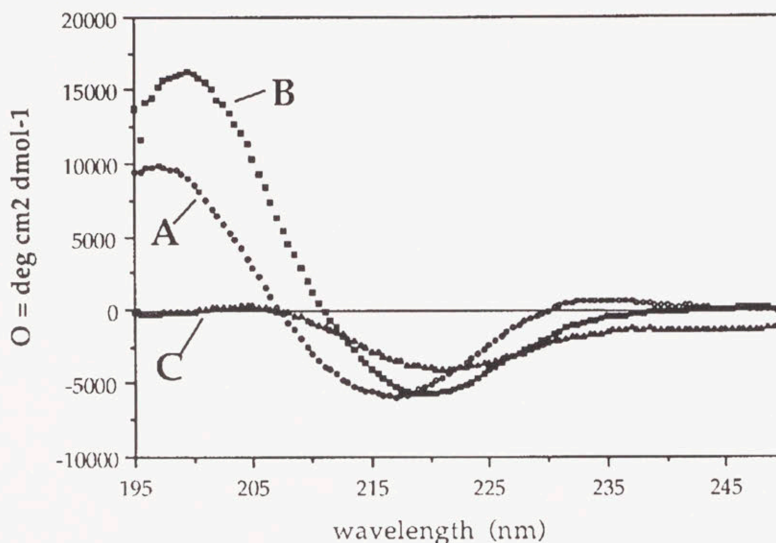


Figure 10. 50 uM ATIII(123-139) + 1 mM **A**. heparin **B**. chondroitin 6-sulfate **C**. dextran sulfate

The ProSec structure assignment program was applied to the spectra of each of the ATIII(123-139) polysaccharide complexes. ProSec is the program installed on the AVIV CD spectrometers. The method is based on that of Chang et al., using a reference set of 16 proteins of known structure.[6] As shown in Figure 10, the spectra only extented to 195 nm. The program was allowed to run the necessary cycles (15) until the "best fit" was determined. The following chart summarizes the results of the program.

Structure	Fractional Composition		
	heparin	chondroitin 6-sulfate	dextran sulfate
α-helix	8%	23%	6%
β-sheet	80%	35%	51%
β-turn	9%	42%	19%
random	3%	0%	23%

The results of the program were consistent with our interpretation that the heparin complex resembles a β-sheet rather than an α-helix. The other two complexes were also decomposed by the program to contain primarily β-sheet or β-turn structure.

FTIR could have helped verify the identity of the structures observed in the CD spectra.

FTIR is another spectroscopic technique which has been applied to the study of protein and peptide secondary structure.[2, 15] The amide I stretch is sensitive to changes in the hydrogen bonding of the amide bond carbonyl. In a random conformation, the amide bond carbonyl is hydrogen bonded to solvent, while in the α-helix or the β-sheet, this carbonyl is involved in either intra-molecular or intermolecular hydrogen bonding. Peptides and proteins in the b-sheet conformation display a marked shift in their amide I stretch as compared to the "random" conformation. The amide I stretch of a β-sheet occurs characteristically at 1630 cm^{-1} rather than the "random" conformation stretch centered at 1650 cm^{-1}.

The FTIR spectra of heparin and related sulfated polysaccharides are dominated by a large stretch at approximately 1230 cm^{-1} corrsponding to the sulfates. The N-acetyl glucosamine amide I stretch is typically found at 1620 cm^{-1}. In theory one should be able to observe the peptide amide I in the presence of the heparin.[15] FTIR of aqueous samples are usually run in D_2O to avoid interference with the fine structure of H_2O. To obtain a good signal to noise ratio, FTIR samples are typically run with very short pathlengths (0.15 mm) and fairly concentrated samples (e.g. > 1mM peptide). FTIR spectra of the peptides alone were easily obtained (see Appendix). However, upon trying to prepare samples of the peptide-polysaccharide complexes it was found that the solubilities, using either peptide, were very limited. Above a peptide concentration of 0.1 mM, added polysaccharide caused a precipitate form. A number of different strategies were tried to get the complexes into solution at higher concentrations. The precipitate was insoluble in all organic solvents tried (DMSO, DMF, HFIP, Acetic Acidic.) Ultimately, thin films were prepared of the dilute samples and used directly for FTIR. However, the peaks obtained were extremely broad and no interpretation could be made.

The same solubility problems which plagued the use of FTIR, prohibit the use of NMR spectroscopy. Two-dimensional NMR experiments were out of the question. Even

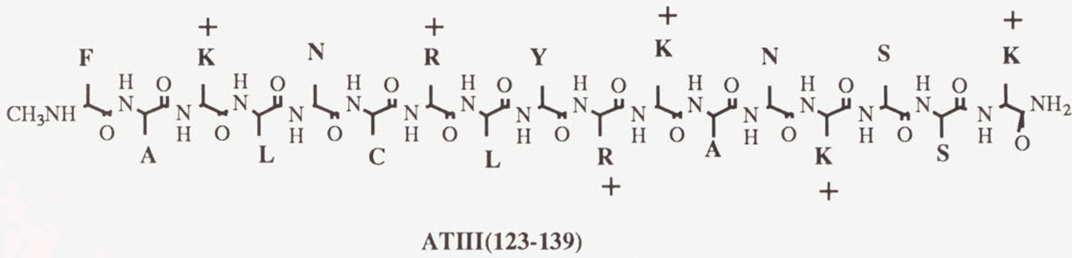
attemps at getting a standard ^1H spectrum of the complex continuously resulted in precipitation. The filtrate showed only heparin signals in the NMR spectrum.

Discussion and Conclusion

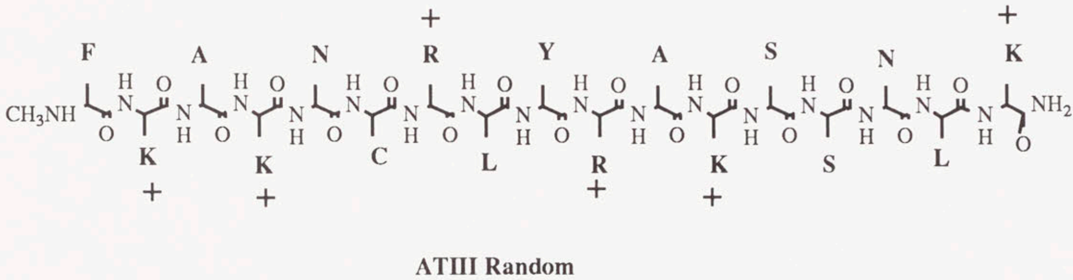
Three peptides were studied by CD spectroscopy in both the absence and presence of heparin. Each of the peptides had no stable secondary structure in a variety of aqueous solutions. However, each peptide could assume α-helical conformation in TFE or SDS. In the presence of heparin, the two peptides derived from the native protein sequence, ATIII(123-139) and ATIII N 135 GlcNAc, assumed a stable secondary structure, while the conformation of sequence permuted analog peptide, ATIII Random, was unaffected. The stable structure induced in the peptides by heparin strongly resembled that of a β-sheet rather than the predicted α-helix. Chondroitin 6-sulfate and dextran sulfate, two sulfated polysaccharides having negligible anti-coagulant activity, were also found to induce structure in ATIII(123-139) but not in ATIII Random. No difference in behavior was seen between ATIII(123-139) and ATIII N135 GlcNAc in either buffer or TFE, while minor differences were seen in each of the two peptide-heparin complexes.

Many reports of peptide β-sheet or β-strand conformation induced by a peptide-ligand interaction involve amphiphilic peptide sequences.[27] For example, peptide sequences derived from the opioid peptide dynorphin have been shown to assume β-strands in the presence of phospholipids.[28] An amphipathic β-sheet forming sequence is usually characterized by alternating hydrophobic and hydrophilic amino acids. When such a sequence assumes the extended β-sheet structure, all the hydrophobic side chains will be found on one face of the sheet or strand, and the hydrophilic residues on the other.

If the peptides ATIII (123-139) and ATIII Random are displayed in an extended β-strand conformation, one finds that the there is no clear segregation of hydrophobic and hydrophilic residues.



ATIII(123-139)



ATIII Random

The more surprising finding is that the peptide sequences are remarkably similar in their charged residue distribution when displayed as an extended β-strand.

In 1989 (a year after our project was initiated) Cardin and Weintraub published a review article in which they compared the sequences of 49 putative heparin binding sites from 21 proteins (e.g apolipoprotein B, vitronectin, and basic fibroblast growth factor)[29] They found that all the sequences contained a substantial number of basic residues (Lys, Arg, but rarely His) as well as hydrophobic residues. From the sequence comparisons, they proposed a heparin binding consensus sequence with either of the two following motifs:

[-X-**B-B**-X-**B**-X]

[-X-**B-B-B**-X-X-**B**-X-]

where B represents a basic residue (lys, arg) and X, a "hydropathic" residue. The authors present the region including residues 125-142 as the heparin binding site of Antithrombin III. Residues 130-138 are identified as containing the consensus sequence.

X-**B-B-B**-X-X-**B**-X

130 L-Y-**R-K**-A-N-**K**-S 138

The authors propose that the consensus sequences in general, and specifically that of ATIII, are likely to form amphipathic helices either by themselves or in the presence of heparin. However they do discuss that in Apolipoprotein E, heparin seems to induce a β-strand, a structural motif that has also been observed in homopolymers of (lys-tyr)n in the presence of heparin. They remark that "heparin may increase the β-strand or α-helical character depending on the precise organization of basic and non-basic residues in the natural peptide sequences". It is also interesting to note that that the sequence of ATIII they chose fit to the motif encompasses the N135 glycosylation site. Many Asn glycosylation sites exist in β-turns.

The peptides we prepared, ATIII(123-139) and ATIII Random, are a test for the Cardin and Weintraub motif. The sequence of ATIII Random was chosen to minimize the possibility of forming a helix with charges segregated to one face. However, a portion of this anti-amphipathic helix sequence, fits the heparin binding motif as well (as poorly) as the native sequence.

X-**B**-**B**-**B**-X-X-**B**-X

F-**K**-A-**K**-N-C-**R**-L ......

Two reports in the literature this year have attempted to test the heparin binding and conformational properties of synthetic peptides modeled after the Cardin and Weintraub proposal.[30, 31] Peptides derived from a putative heparin binding site on the von Willebrand factor did not undergo a significant conformational change in the presence of heparin.[30] In a second report a peptide was designed containing only alanine and arginine residues.[31] This peptide was reported to be 70 % helical in solution in the absence of heparin, and 100 % helical in the presence of heparin.

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Chapter 4. Experimental Methods

Materials and Supplies

Peptides were synthesized and purified as described in Chapter 3 of this thesis. Ultra-Pure Guanidine-HCl was purchased from ICN Biomedical. Heparin (porcine intestinal mucosal sodium), chondroitin-6 sulfate (shark cartilidge sodium salt), and dextran sulfate were purchased from Sigma.

Concentration of Stock Peptide Solutions

A stock peptide solution was prepared by dissolving lyopholized peptide (~ 5 mgs) in 1-2 mls. of fresh Milli-Q H_2O. 50 uls of stock solution was added to 950 uls of freshly prepared 6M aqu. GdnHCl. The UV spectrometer was blanked with 50 uls H_2O + 950 uls GdnHCl in a scan from 210-300 nm. The cuvette (1 cm pathelength, low volume) was rinsed and dried between the blank and sample scans. The sample was scanned from 210-300 nm and the absorbance at 276 nm was noted. The concentration of the peptide stock solution was calculated using ε= 1470.

Uronic Acid Assay for deterimining heparin /chondroitin sulfate concentrations

The following assay is taken from Bitter and Muir,1964.

Reagents:

A. 0.025 M Borax (sodium tetraborate-H2O) in conc. H_2SO_4.

B. 0.125% carbazole in absolute methanol

C. Glucuronolactone standards ~4-40 ug/ml in H_2O saturated with benzoic acid.

D. H_2O saturated with benzoic acid(for diluting samples).

E. Samples dissolved in H_2O saturated with benzoic acid.

Assay Procedure:

1. 3 mls. of Borax/H2SO4 were added to a 10 mls Kimax tubes w/ screw cap.
2. Tubes were placed in dry ice/acetone and their contents frozen.

3. 0.5 mls. of standard or sample was layered on top of the frozen acid. With the capss tightly in place, the tubes were gently vortexed and placed on regular ice. When the acid was mostly melted, but still cold, the tubes were vortexed again to insure complete mixing.

4. The samples/standards were heated on an oil bathe at 100 °C for 10 minutes.

5. The samples/standards were cooled on ice to room temperature.

6. 100 ul. of 0.125% carbazole were added to each sample/standards and the samples/standards subsequently vortexed.

7. The samples/standards were heated at 100 °C for 15 minutes.

8. The tubes were cooled on ice to room temperature.

9. The UV absorbance of each sample/standard was read at 530 nm.

10. The standard absorbances were plotted against the molar conc, of glucoronolactone using Cricket Graph. A best fit line was determined using the curve fitting menu of Cricket Graph. The sample concentrations were determined by using the linear equation fit for the standards to calculate the molar concentration of uronic acid in the sample.

Additional Comments:

* Carbohydrate samples were diluted into the benzoic acid/H_2O when possible.

* All the H_2SO_4/Borax solution is very viscous! Thorough mixing at every step of the assay is critical for getting a good standard curve and accurate concentrations for the samples.

* Standards and Samples were run in duplicate.

Circular Dichroism Spectroscopy.

CD spectra were obtained on an AVIV 60DS spectropolarimeter in the laboratory of Dr. Robert Sauer, Department of Biology, MIT. A strain free quartz cells of 0.1 cm pathlength borrowed from Kemp group was purchased from Hellma. Samples were

prepared by diluting aliquots of peptide or polysaccharide stock solutions into degassed solvents. Samples were scanned 4 times from 250-195 nm in 0.5 nm steps with 1.0 sec averaging. Individual wavelengths were recorded for 90 seconds in 1 second intervals and the signal averaged over this time. Solvent backgrounds were subtracted from all data. All spectra were obtained at 25° C and have been generated in triplicate.

Digitized data (MS-DOS) from the CD spectrometer was transfered to Cricket Graph using the Apple File Exchange. The data was converted from millidegrees to mean residue ellipticity using the following equation:

$$[\theta] = \text{mdegs.} / \text{mmolar concentration} * 1/\text{pathlength} * 1/\text{chain length} * 100$$

Data was smoothed using the smoothing function of Cricket Graph with a 5 point smooth.

Fourier Transform Infrared Spectroscopy.

Solution FTIR spectra were obtained in 99.9% D_2O in 0.15mm pathlength CaF_2 cells on a Mattson Cygnus 100 FTIR spectrophotometer at the Spectroscopy Laboratory of the Department of Chemistry at M.I.T. Spectra were corrected for solvent background.

N-Deacetylation of Heparin

The following procedure was taken from Shaklee and Conrad, Biochemical Journal (1984), 217, 187-197.

46 mgs. of heparin sodium salt (porcine intestinal mucosa), 6 mgs. hydrazine sulfate, and 600 uls anhydrous hydrazine were heated for 4 hours at 100 ° C. The reaction mixture was dried under vacuum. The dried product was taken up once in ethanol and dried, and once in $H_2O$and dried, before being lyopholized from H_2O.

The lyopholized product was dissolved in 1.5 mls. 0.25 M aqueous HIO_3 . The solution immediately turned black. The solution was washed with diethyl ether (4x) until both the aqueous and ether layers were colorless. The aqueous layer was passed through

a pre-packed Biogel P-10 column in H_2O to remove any residual HIO_3. The first 3 of 12 collected fractions were pooled and lyopholized. 9 mgs of heparin was recovered (20% yield based on starting material). ^{1}H-NMR in D_2O confirmed that the deacetylation was complete. The lyopholized product was used directly for CD experiments.

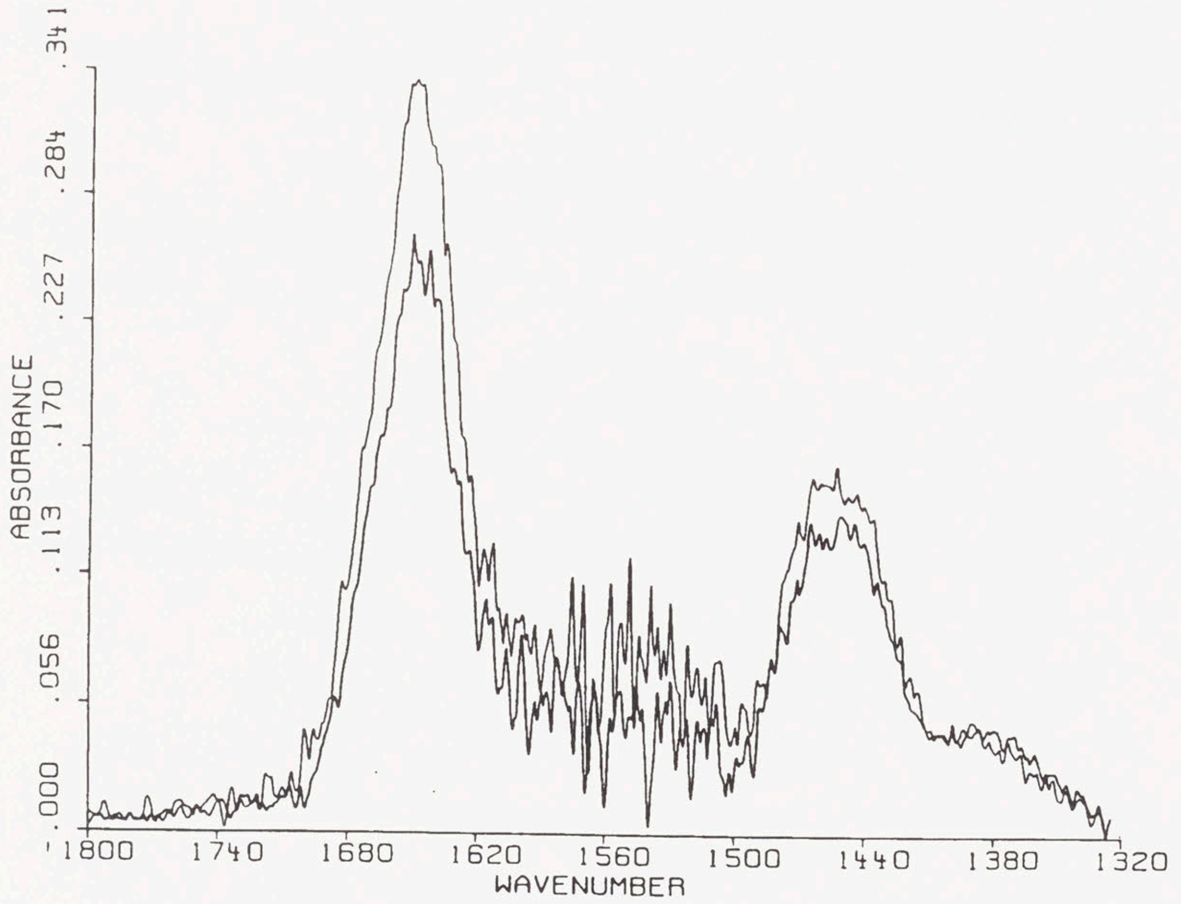
Chapter 4 . Appendix

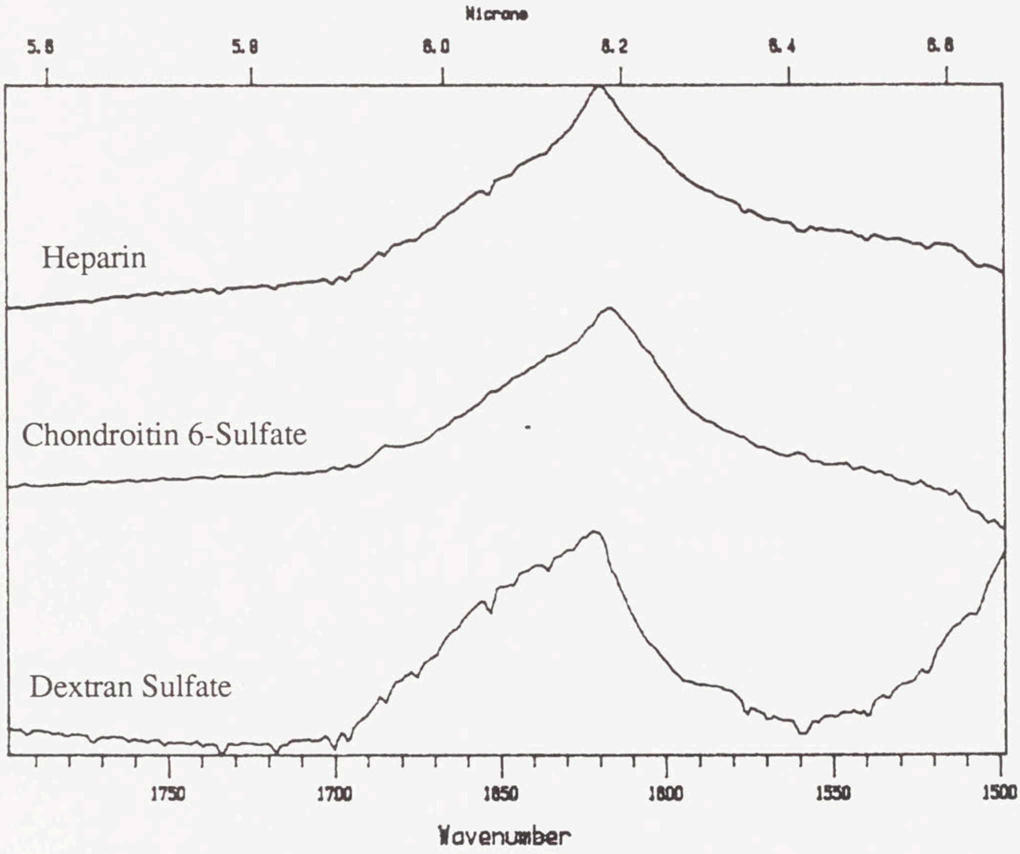
FTIR of ATIII(123-139) and ATIII Random in D_2O.

FTIR of Thin Films of ATIII(123-139)-polysaccharide complexes.

Crude and Deacetylated Porcine Heparin in D_2O.

FTIR of ATIII(123-139) and ATIII Random in D_2O




Thin Films of ATIII(123-139) + Polysaccharides
Microns
Heparin
Chondroitin 6-Sulfate
Dextran Sulfate
1750
1700
1650
1600
1550
1500
Wavenumber

Crude Porcine Heparin in D_2O

6.0 5.0 4.0 3.0 2.0 1.0 PPM 0

De-N-acetylated Heparin in D2O

6.0 5.0 4.0 3.0 2.0 1.0 PPM

Chapter 5
Binding Properties of the Peptide-Polysacharide Complexes

Introduction

Antithrombin III binds crude heparin with a K_d of 1 x 10^{-7} M, resulting in a change in protein conformation. Any model for this interaction should therefore mimic both the binding affinity and the conformational change. In the previous chapter we showed that peptides based on the native protein sequence, ATIII123-139 and ATIII N135 GlcNAc, were capable of undergoing conformational changes in the presence of heparin, while a sequence permuted analog was not. The work described in this chapter was directed towards characterizing the heparin-peptide binding interaction and determining how this interaction compares to the heparin-ATIII binding interaction. The stoichiometry of the ATIII(123-139) polysaccharide complexes, the sensitivity of the binding event to salts, and the dissociation constants for the peptide-heparin interactions were determined. Additionally, two experiments were performed to explore the possibility that the peptide ATIII(123-139) displays any heparin sequence specificity in its binding.

Heparin is heterogeneous at the level of chain length (MW 5,000-25,000) and monosaccharide composition.[2] Heparin polysaccharides can therefore be described as ensembles of different (unequal) binding sites within lattice-like structures. Binding to

lattice of similar but non-identical binding sites is extremely complex.[3] If one had homogeneous, well characterized heparin fragments, any binding event would be greatly simplified. Having only native heparin at our disposal, only the most simple binding experiments were performed to determine macroscopic properties of the heparin-peptide interactions.

Complex Formation is Saturable

A series of titration experiments were performed in order to ascertain that the CD signals observed for the ATIII(123-139)- polysaccharide samples result from the formation of a discrete complex. These experiments provide an indication of the peptide to polysaccharide ratio required to induce structure in the peptide. They may also reveal the frequency of occurence of the peptide binding site withih the heparin sequence.

Two different titration experiments were run. In the first set of titrations, the samples contained a fixed quantity of polysaccharide and varying quantities of peptide. An example of such a titration is shown in Figure 1.

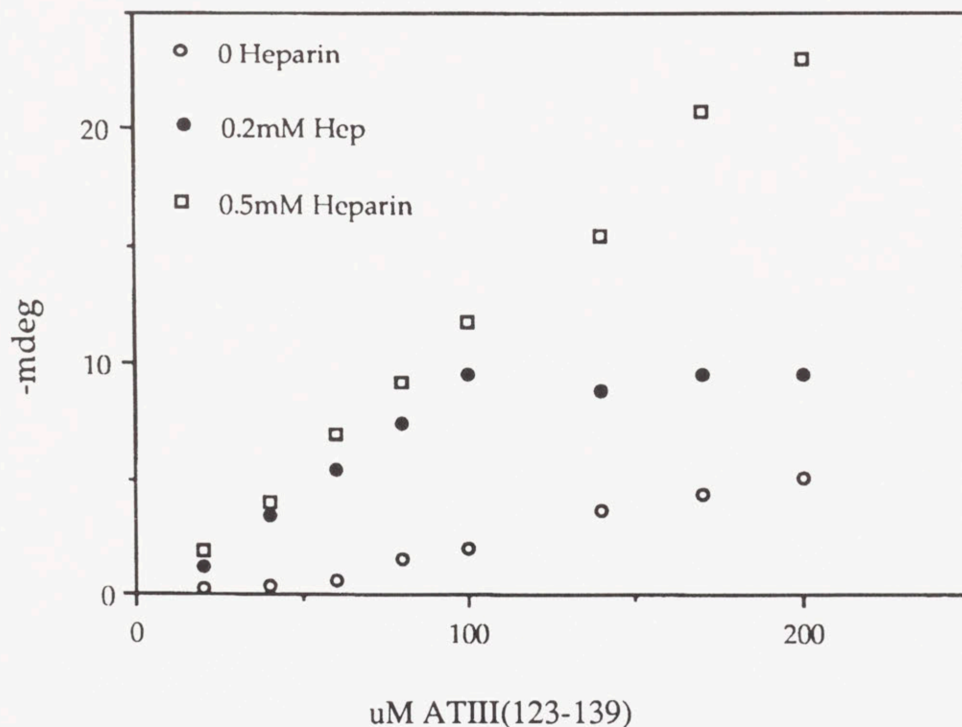


Figure 1. Three sets of samples in 50 mM Phosphate, 50 mM NaCl pH 7. The heparin concentrations are in M uronic acid which equals M disaccharide.

In all the experiments reported in this chapter, polysaccharide concentrations are reported in saccharide concentration because the polysaccharide chain lengths are heterogeneous. An assay for uronic acid content was used to quantify the heparin and chondroitin 6-sulfate stock solutions.[4] Dextran sulfate stock solutions were determined by weight.

For the samples containing 0.2 mM heparin uronic acid (0.4 mM heparin saccharides), the signal saturated at 100 uM peptide, or 4 saccharide residues per peptide. When the heparin content of the samples was increased to 05 M uronic acid heparin (1.0 M heparin saccharides), the CD signal continued to increase. This trend demonstrates that the peptide must bind polysaccharide in order for the conformational change to take place. This point is important in light of the possible β-sheet nature of the observed complex. The saturation at lower heparin concentration rules out the possibility that the heparin serves to nucleate β-sheet formation in a large number of peptides.

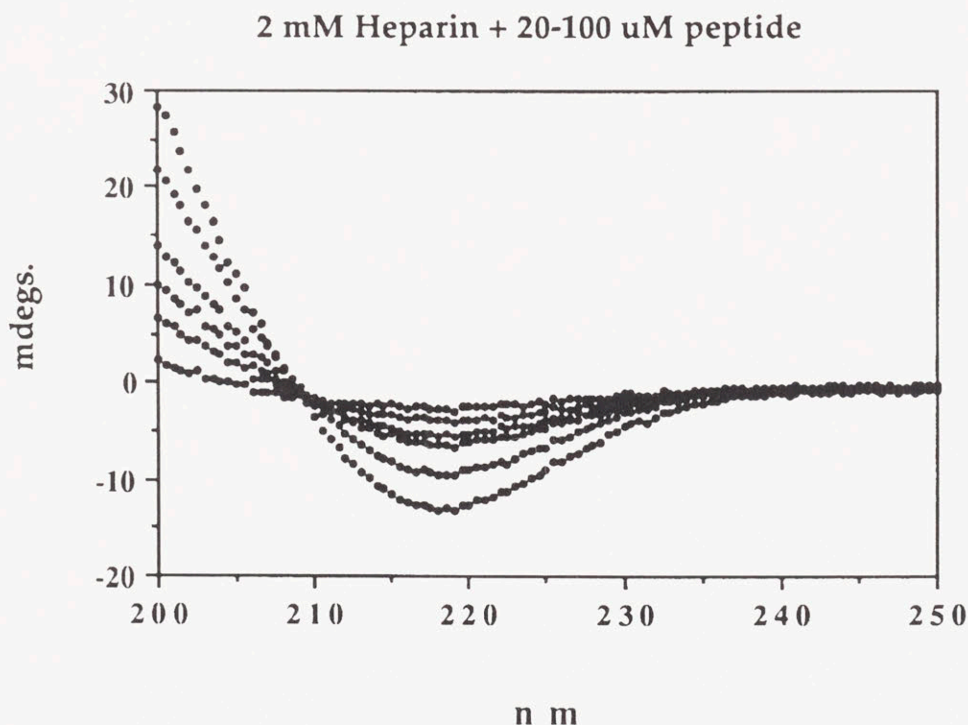


Figure 2. CD scans of samples containing 4 mM Heparin and 20,30,40,50,75 and 100uM ATIII(123-139) in 50 mM Phosphate, 50 mM NaCl, pH 7.

The CD scans presented in Figure 2, complement the titration shown in figure 1. Again samples were prepared with a constant quantitiy of heparin (4 mM saccharides) and varying quantities of peptide (20um-100uM). The well defined isodichroic point of the superimposed scans demonstrates that the same structure is growing as more peptide is bound to the heparin.[5] This finding indicates that a two-state binding phenomenon is being observed.

A second set of titration experiments was performed in which the peptide concentration was held constant and the concentration of polysaccharide was varied. In these experiments the CD signal was also found to saturate. The concentration of polysaccharide at which the CD signal reaches a maximum is used to estimate of the number of saccharides necessary to induce structure in a given quantity of the peptide. The results of these polysaccharide titrations are shown in figure 3.

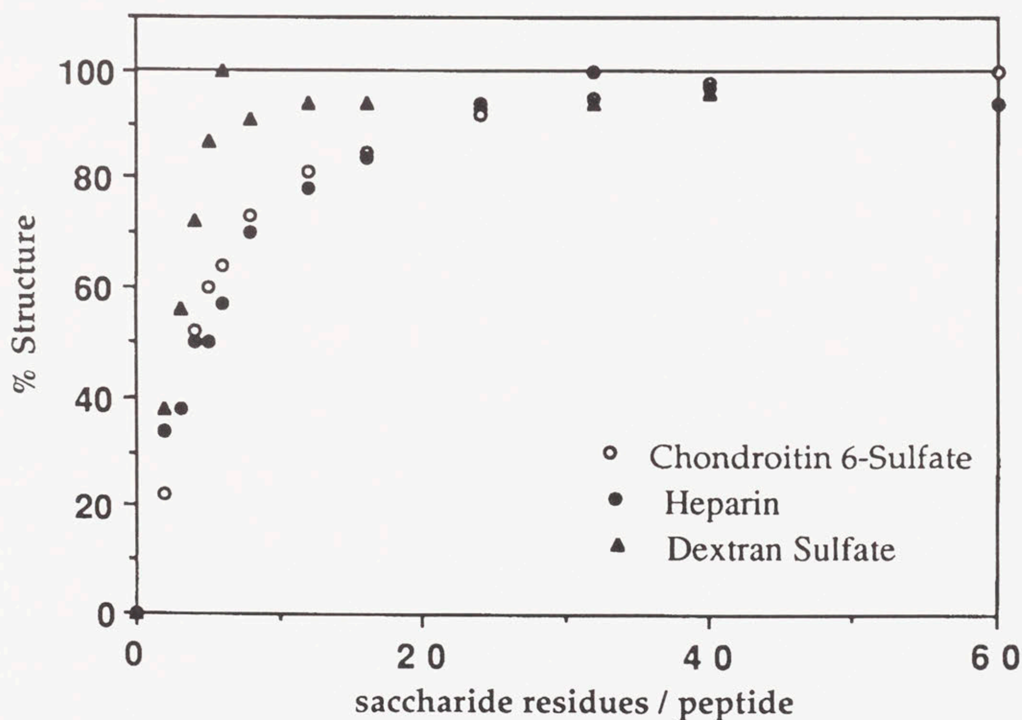


Figure 3. Samples containing 50 uM ATIII(123-139) and 0-3mM(residue) polysaccharide in 50 mM phosphate, 50 mM NaCl, pH 7.

The dextran sulfate-peptide interaction formed maximal structure at a 8:1 saccharide to peptide ratio. Chondroitin 6-sulfate and heparin showed maximal structure formation at a stoichiometry of 20:1, saccharide to peptide. The lower stoichiometry of

the dextran sulfate relative to the other two sulfated polysaccharides may result from the fact that the dextran sulfate polymer is a 1-6 linked polysaccharide chain while heparin is linked 1-4 and chondroitin 6-sulfate alternates between 1-3 and 1-4 linkages. The dextran sulfate chains therefore may be more flexible than the other two types. Dextran sulfate is also the most homogeneous substance of the three compounds.

It was hoped that this experiment (constant peptide, variable polysaccharide) might give some indication of whether the ATIII(123-139) peptide binds an uncommon heparin sequence as does human ATIII. If the structure induced in the peptide results from binding to a rare heparin sequence (assuming also that the affinity for that sequence is several orders of magnitude higher than any other binding sites) then the CD signal would be expected to saturate at a very high polysaccharide to peptide ratio. For example, the ATIII binding site occurs approximately once on every third native chain (100 saccharides long).[2] Therefore, a saturation point on the order of 300 saccharides per peptide may have indicated very specific binding. The stoichiometry of the ATIII(123-139)- heparin complexes gave no indication of binding to a rare heparin sequence.

<u>The Complex is Sensitive to the Ionic Strength of its Environment</u>

The complex is sensitive to changes in ionic strength

Some protein-ligand binding interactions are sensitive to changes in the ionic strength of their environment.[6] Such sensitivity is an indication that electrostatic interactions contribute significantly to the binding phenomenon. Raising the ionic strength of the solvent decreases electrostatic attractions or repulsions between ions.[7] Because heparin is highly anionic and the peptides highly cationic, the binding interaction described herein is likely to be electrostatic in nature. Therefore, increasing the ionic strength of the solvent should disrupt binding.

Two different types of experiments were performed to explore the effects of salt on the peptide-polysaccharide complexes. Initially, samples of the ATIII(123-139)-polysaccharide complexes at various NaCl concentrations were monitored using CD

specotroscopy. Each complex was monitored at its characteristic negative signal (217 nm heparin, 222 nm for chondtroitin 6-sulfate and dextran sulfate) Full CD scans,(250-200) nm, were also obtained of the samples to ensure that a one step dissocation was being followed. (See Figure 4).

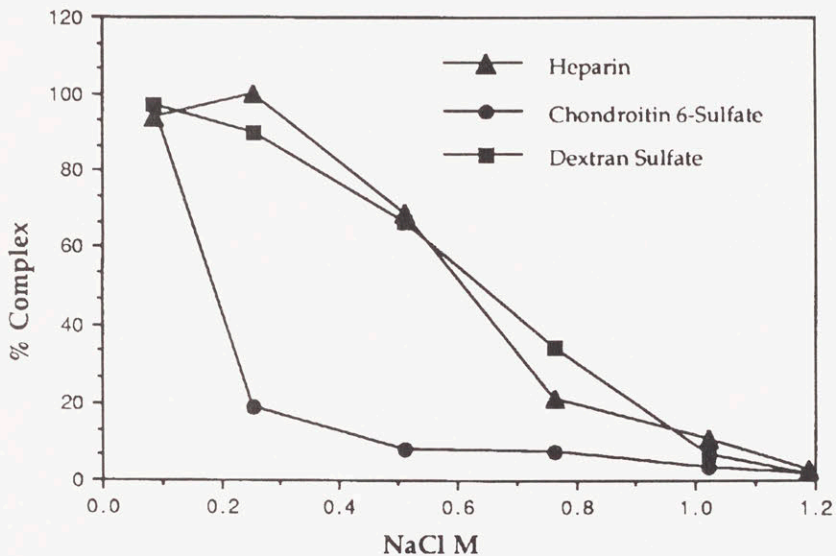


Figure 4. NaCl dissociation of ATIII(123-139)-polysaccharide complexes

In the CD stucture monitoring experiments, heparin and dextran sulfate gave similar results, while chondtroitin -6-sulfate was found to be more sensitive to salt than the others. A concentration of 0.6 M NaCl is necessary to dissociate 50% of the ATIII(123-139)-heparin and ATIII(123-139)-dextran sulfate complexes, while only 0.2 M NaCl is needed to dissociate 50 % of the chondroitin 6-sulfate complex. This finding is consistent with the fact that chondoitin-6-sulfate has only one sulfate residues per disaccharide while dextran sulfate has two and heparin, on average, has 2.5 .

Commercially available heparin-agarose was used to compare the heparin affinity of ATIII(123-139) with that of ATIII Random and ATIII N135 GlcNAc.(figure 5) ATIII(123-139) and ATIII N135 GlcNAc are eluted from the column over a range of 0.3-0.6 M NaCl, while ATIII Random elutes completely from the column in less that 0.3 M NaCl. An interesting feature of the elution profiles is that ATIII Random consistently elutes in a sharp peak while ATIII(123-139) elutes in a broad band over a wide range of

NaCl concentrations. The same trend is observed whether the NaCl gradient is run in steps or as a continuous linear gradient. The broad elution profiles of ATIII(123-139) and ATIII N135 GlcNAc indicates that these peptides may bind several sites within heparin.

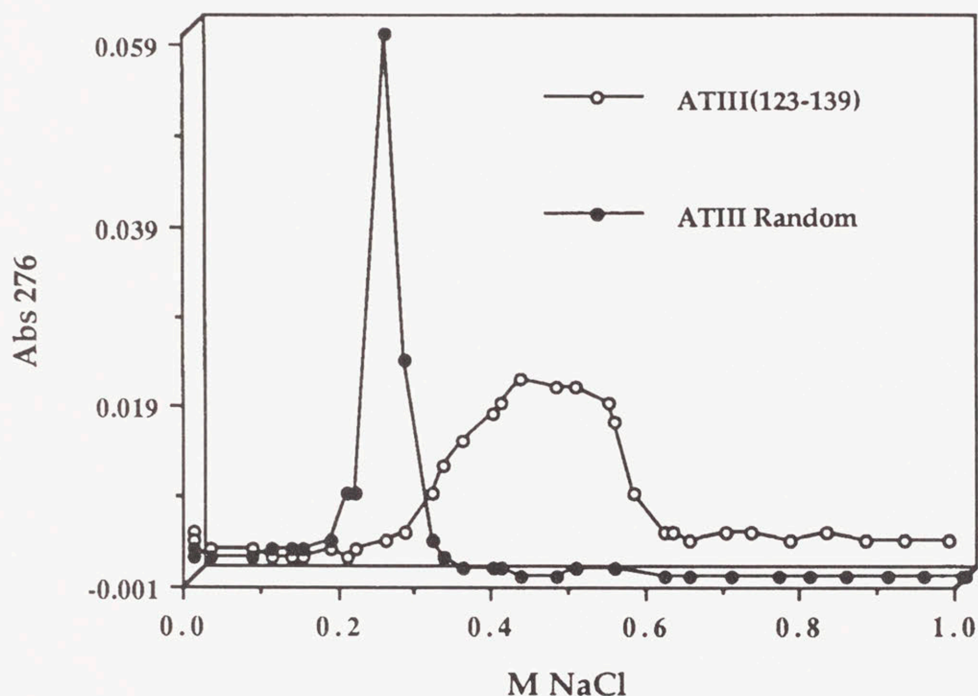


Figure 5. NaCl gradient elution profiles of peptides applied to heparin-agarose

The results of the CD and heparin agarose experiments indicate that the amount of NaCl needed to fully disrupt structure is higher than the amount of NaCl needed to disrupt binding. At 0.6 M NaCl only 50 % of the structure observed in the ATIII(123-139) heparin complex is disrupted, while at 0.6 M NaCl in the elution profile, ATIII(123-139) is completely dissociated from heparin-agarose. The discrepancy between the two experiments may reflect a situation in which only a small portion of peptide tightly bound to heparin is responsible for the observed CD signal. A more plausible explanation for the discrepancy is that the heparin-agarose experiment perturbs the binding equilibrium while the CD experiment does not. The amount of peptide eluting from the column may not accurately represent the quantities bound to heparin at the given conditions. However, because ATIII Random has no induced CD signal in the presence of heparin, the

behavior of ATIII(123-139) and ATIII Random can only be directly compared using the heparin agarose experiment.

A Chaotropic Salt has no special effect on the ATIII(123-139) -heparin interaction

Chaotropic salts (LiClO4, NaSO4, GdnHcl) disrupt hydrophobic interactions. Solvation of a hydrophobic molecule causes increased order in the surrounding water at a high entropic cost ($-\Delta S$).[7] Hydrophobic interactions are driven by the need to minimize this unfavorable entropic effect. Chaotropic salts disorder water, thereby reducing the drive for hydrophobic bonding or interactions.[7] Sensitivity of a binding or structural event to chaotropic salts may indicate that hydrophobic effects are important to the interaction. The calicheamycin-DNA interaction, for example, has been shown to be more sensitive to chaotropic salts than to NaCl.[8] Although heparin contains many sulfates, the conformation of a heparin chain may allow for both hydrophobic than hydrophilic faces. It was determined that the elution profile of ATIII(123-139) was identical whether LiClO4 or NaCl was used as the eluting salt. This finding indicates that hydrophobic interactions do not contribute significantly to the ATIII(123-139)-heparin interaction.

The ATIII(123-139)-Heparin Complex is Very Sensitive to $CaCl_2$

Heparin binding to divalent cations such as Mg^{2+},Ca^{2+}, Cu^{2+} and Zn^{2+} has been studied by an number of researchers.[9, 10, 11] Heparin can distringuish amonst the cations listed. Cations such as calcium are thought to be chelate between residues along a chain.[12, 13] It has been proposed that calcium binding limits or changes the conformation of the polysaccharide chain.[13] Literature reports on the influence of Ca^{2+} in the ATIII-heparin interaction are conflicting.[14]

Using the same heparin-agarose elution experiment as presented previously, the ATIII(123-139) complex was found to be more sensitive to $CaCl_2$ concentration than NaCl concentration. The peptide was completely eluted by 0.2 M, corresponding to the ionic stength of 0.4 M NaCl. In the NaCl elution experiment, the peptide eluted between

0.3 and 0.6 M NaCl. The CD spectrum of the ATIII(123-139)-heparin complex in small (20 mM) concentrations of CaCl2 is substantially different from that containing only NaCl.

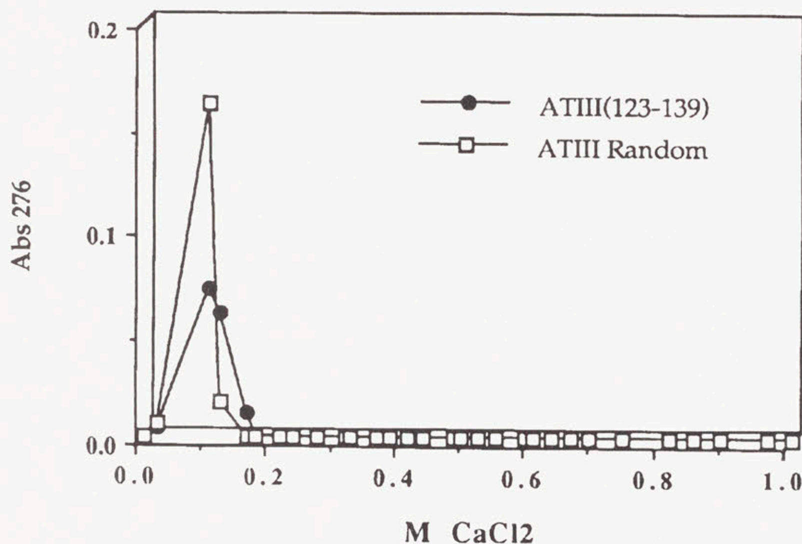


Figure 6. Elution profile of ATIII(123-139) and ATIII Random in $CaCl_2$

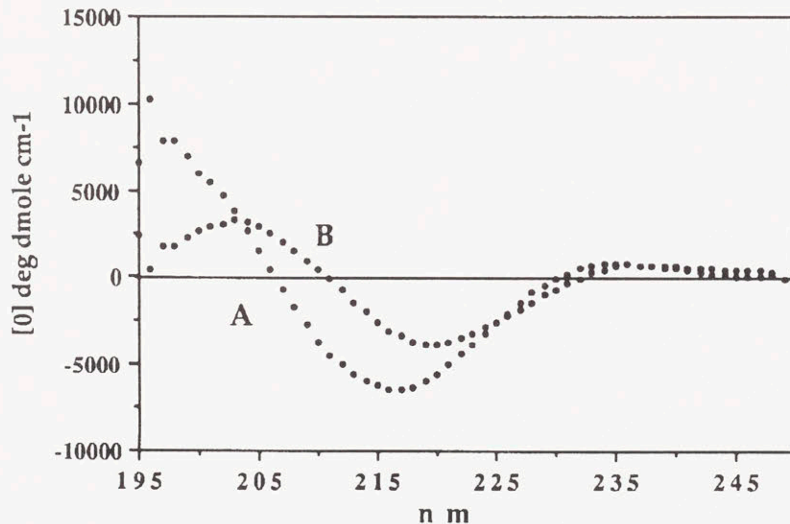


Figure 7. A. 50 uM ATIII + 1 mM Heparin in 150 mM NaCl
B. 50 uM ATIII + 1 mM Heparin in 110 mM NaCl + 20 mM $CaCl_2$.

The differences in the ATIII-heparin complex CD spectra in the presence and absence of 20 mM $CaCl_2$ may result from of several diferent factors. First, ATIII(123-139) and Ca^{2+} may compete for the same anionic binding sites. Second, Ca^{2+} may alter

the conformation of the heparin in such a manner as to perturb the binding of ATIII(123-139) to heparin.

Dissociation Constants for the Peptide-Heparin Complexes

A direct way to compare the binding of each of the peptides to heparin is to determine a dissociation constant. A dissociation constant allows one to put the behavior of the peptides described in this project in context with other heparin binding peptides and proteins, as well as other peptide-ligand interactions.

The structure induced in ATIII(123-139) by heparin could not be ued to determine a binding constant. The concentrations of peptide and polysaccharide required to observe the CD signal are high enough to represent a stoichiometric binding event. It is difficult to extract binding constants from stoichiometric conditions. [15] We also wanted to make the distinction between the properties that define the structure formation and those that define the binding affinity. Additionally, because ATIII Random has no induced CD signal in the presence of heparin, CD could not be used to compare the behavior of ATIII Random and ATIII(123-139).

Dissociation constants for heparin binding proteins have been determined using a number of different methods. Rosenberg *et al.* used fluorescein labeled heparin fragments in an assay where protein binding was measured as a function of the increased fluorescence of the labeled heparin fragments.[16] ^{1}H-NMR spectroscopy has been used by Gettins et al. to determine a binding constant based one the chemical shifts of histidine residues in ATIII upon binding to heparin.[1] Another technique was developed by Lander *et al.*, using agarose gels impregnated with a various concentrations of heparin.[17] A binding constant is determined as a function of the rate at which a protein moves through the gel under an electric field.

We chose to use a filter binding assay, not unlike a DNA hybridization assay, to determine the binding constant. This binding assay was developed by Repligen to monitor the binding of growth factors to heparin. This assay uses ^{3}H labeled heparin.

Samples are prepared containing ^{3}H-heparin and a known quantity of peptide or protein. The samples are passed through a nitrocellulose filter. The peptide and peptide-heparin complexes adhere to the filter, while the unbound heparin is washed through. The amount of complex formed is determined by the amount of ^{3}H-heparin detected on the filters. Provided the correct concentration range of peptide is chosen and the concentration of heparin is below that of the K_d, a plot of % bound vs peptide concentration will produce a curve in which the peptide concentration at 50% bound represents the K_d.

Commercially available ^{3}H-Heparin is prepared by reduction of the reducing terminal residue with ^{3}H $NaBH_4$.[18] The ^{3}H-heparin is crude sodium salt with a MW range of 6,000 to 20,000. In this assay isn't necessary to know the exact concentration of the heparin. Conseqently the fact the heparin chains are of variable lengths is unimportant.

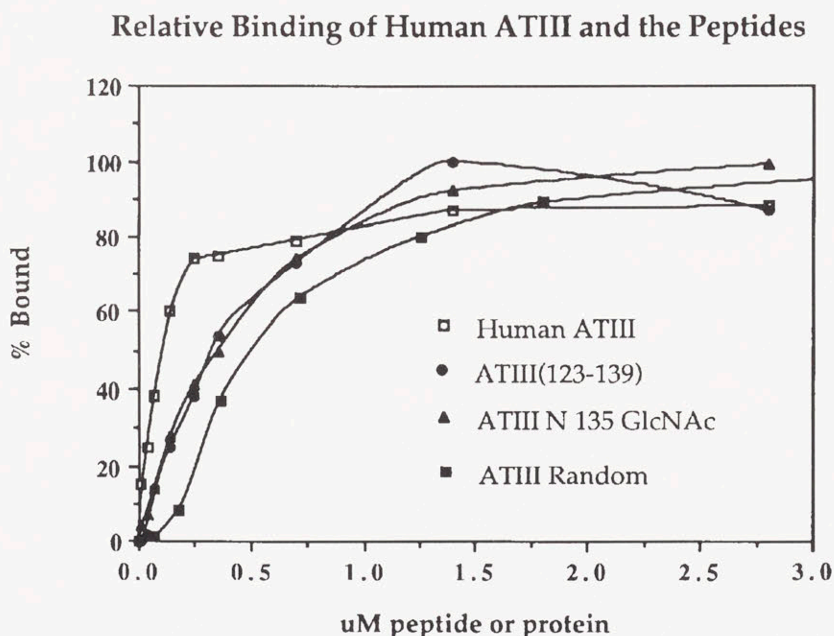


Figure 8. Binding data for human ATIII and related peptides

The assay was first applied to human Antithrombin III to show that the a binding constant consistent with that reported in the literature could be obtained. The assay was

then performed on each of the three peptides. The results taken from Figure 8, are summarized as follows:

	K_d
human ATIII:	1.0×10^{-7} M
ATIII(123-139)	3.5×10^{-7} M
ATIII N135 GlcNAc	3.5×10^{-7} M
ATIII Random	5.4×10^{-7} M

The results indicate that ATIII(123-139) has greater affinity for heparin than does ATIII Random. However, both peptides bind within the same order of magnitude as the whole protein. The GlcNAc residue on ATIII N135 GlcNAc does not alter the heparin affinity of peptide, whereas the native glycoprotein has reduced affinity as compared to a mutant ATIII lacking the carbohydrate at Asn 135.[19]

Can ATIII(123-139) Recognize the Antithrombin III Binding Site on Heparin?

In 1976, Lam et al. showed that the Antithrombin III binds to only one third of the native heparin chains in a given heparin preparation. It was ultimately demonstated that ATIII recognizes a specifc rare pentasaccharide sequence within heparin. To complete our study of the model peptides, we wanted to know if the peptide ATIII(123-139) could also recognize this specific heparin sequence.

A good experiment would have been to obtain the synthetic ATIII binding pentasaccharide and study both the conformational change and the binding behavior of the peptide to this polysaccharide. However, since we did not have access to this material , we approached the question of sequence specific binding in two different experiments. Our first attempt at this question was to assay for competition in heparin binding between the protein and each of the peptides. Our second attempt was to isolate heparin with an increased affinity for the protein and assay for changes in the dissociation constant of the peptide ATIII(123-139).

A Competition Binding Assay

A competition binding assay was developed utilizing the intrinsic fluorescence of human ATIII. The intrinsic fluorescence spectrum of the protein Antithrombin III is enhanced by 30% upon binding to heparin. The fluorescence enhancement has been shown to result from changes in the environment of 4 tryptophan residues buried within the protein. [20] Heparin binding can be monitored by exciting a ATIII-heparin complex at 300 nm and observing the change in fluorescence at 340 nm. The intrinsic fluorescence of the protein was determined for a series of samples containing fixed quantities of heparin and ATIII (200 uM and 3.5 uM respectively) and variable quantities of the peptides (3-35 uM). The fluorescence of the protein in the absence of heparin was designated as "0 % bound". The intrinsic fluorescence of the protein + heparin was designated as "100% bound". The intrinsic fluorscence of each sample containing peptide was converted into an expression of percentage protein bound and plotted against peptide concentration. The results are shown in Figure 9.

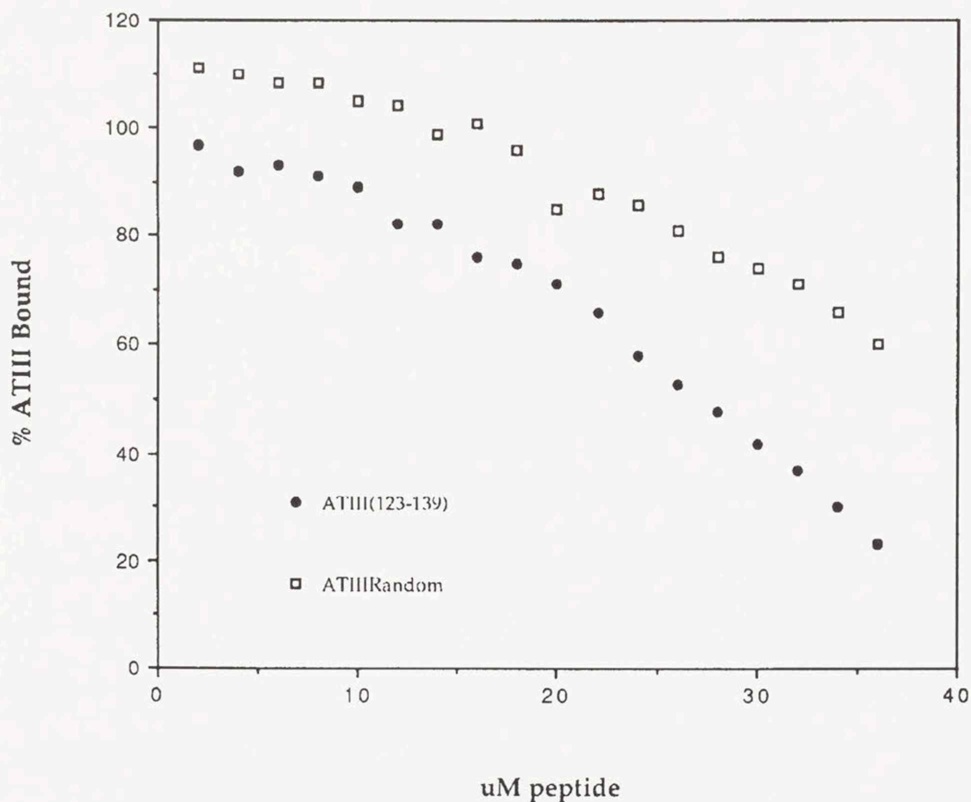


Figure 9. Dissociation of 3.5 uM ATIII from 200 uM heparin as a function of added peptide

The results showed that at 16 uM peptide, 24 ± 2% of the ATIII-heparin complex was dissociated with ATIII(123-139), while little dissociation was observed with ATIII Random. At 36 uM peptide, a 10-fold excess over protein concentration, 77 ± 1% of the ATIII-heparin complex was dissociated in the presence of ATIII(123-139) as compared to 27 ± 2% observed for the samples containing ATIII Random.

At the time this experiment was performed, no other data was available assessing the affinity of each peptide for heparin. It was therefore not possible to discern whether the higher peptide concentrations required to disrupt the ATIII-heparin binding reflected weaker peptide-heparin affinity or less discriminate heparin sequence requirements for peptide binding.

The results of this experiment are consistent with the dissociation constants later determined for the peptides. The heparin dissociation constants of the peptides are within the same order of magnitude as that of the protein. It is therefore not surprising that only a 10 fold excess of the peptide is required is disrupt binding.

<u>Direct Comparison of Binding to "High-Affinity" Heparin</u>

In order to determine whether ATIII(123-139) can recognize the rare oligosaccharide sequence recognized by ATIII, an aliquot of heparin with high affinity for ATIII was isolated. Jordan et al., originally isolated "High affinity" heparin by coeluting human ATIII with an excess of heparin fragments through a gel filtration column at low salt.[21] An ATIII-heparin fragment complex elutes from the column first, followed later by the unbound heparin. The ATIII-heparin complex is applied to a second gel filtration column in high salt eluent that will disrupt the ATIII-heparin interaction. In the second column, ATIII elutes first, followed later by the dissociated "high affinity" heparin. The "high affinity" heparin has increased affinity for ATIII as well as increased activity in enzymatic assays.

We isolated a "high affinity" heparin fraction by applying an aliquot of ^{3}H-heparin to a commercially available preparation of human-ATIII linked to agarose.[22] The

heparin was applied at low salt (0.05 M). The high affinity heparin was isolated from a 1.0 M salt column wash. The "high affinity" heparin isolated was subsequently used in the filter binding assay with human ATIII to demonstrate that the reported increase in protein affinity could be detected. The results are shown in Figure 10.

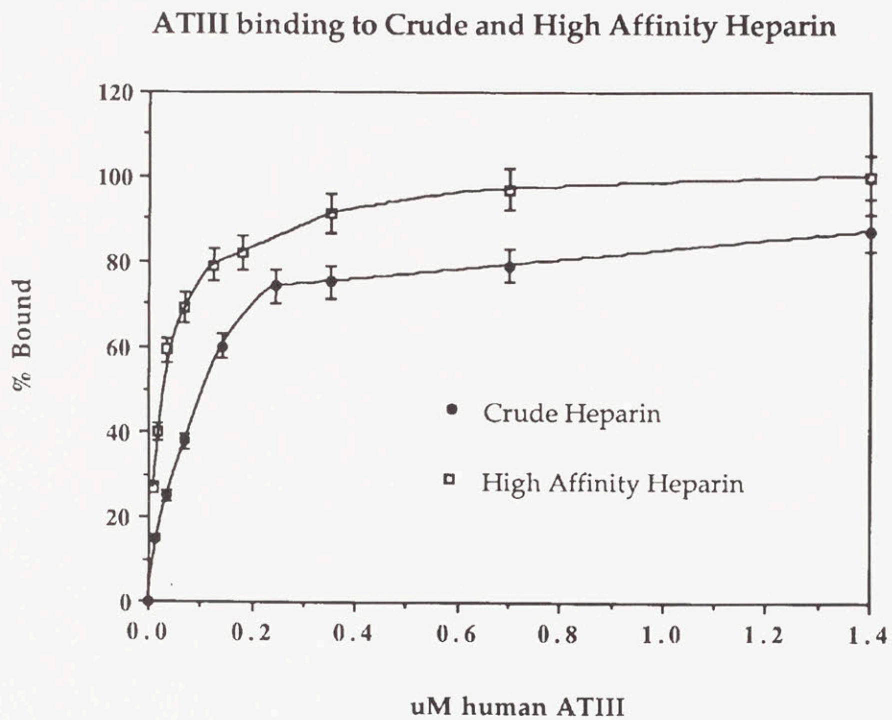
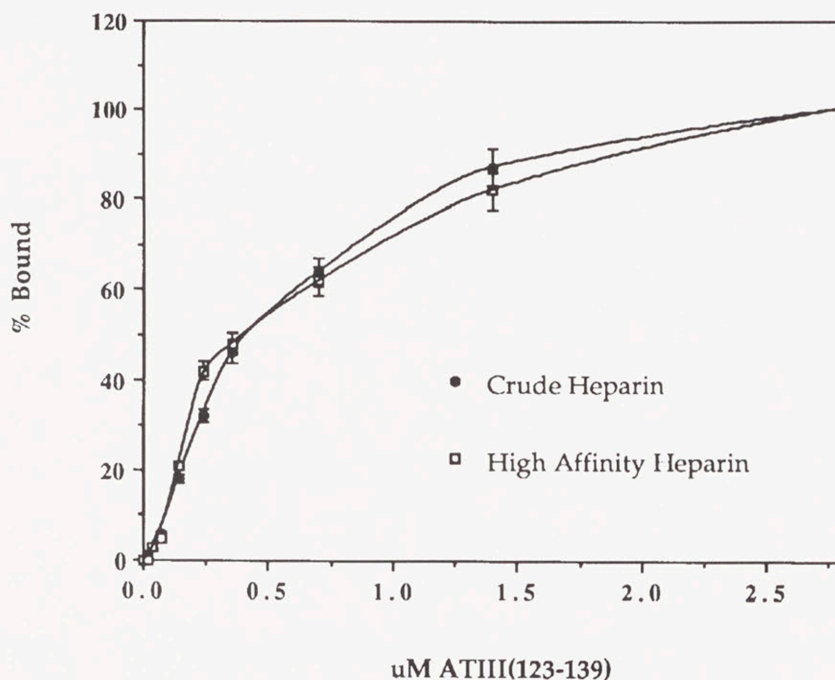


Figure 10. Above: Human ATIII with Crude and High Affinity Heparin

Figure 11. Below: ATIII(123-139) with Crude and High Affinity Heparin



The assay was then applied to ATIII(123-39) and ATIII N135 GlcNAc using both the crude and high affinity heparin preparations. No shift in the binding curve was seen with the high affinity heparin in either peptide. This finding demonstrates clearly that the peptides tested do not bind specifcally to the ATIII binding region of heparin.

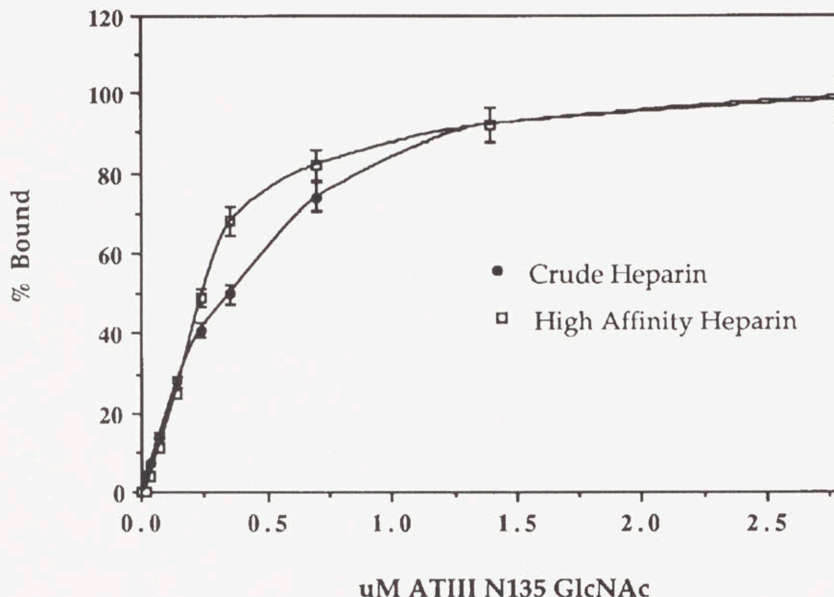


Figure 12. ATIII N135 GlcNAc with Crude and High Affinity Heparin

Conclusions

The structure observed in each of the ATIII(123-139) -polysaccharide complexes was shown to be saturable at saccharide to peptide ratios of 20 to 1 for heparin and chondroitin 6-sulfate and 8-1 for dextran sulfate. The structure of each of the complexes was sensitive to the ionic strength of the solvents. The structure of the ATIII(123-139)-heparin and ATIII(123-139)-dextran sulfate complexes were 50 % dissociated in 0.6 M NaCl, whereas the ATIII(123-139)-chondroitin 6-sulfate was 50% dissociated by 0.2 M NaCl.

The heparin binding properties of ATIII(123-139) and ATIII Random were compared. ATIII(123-139) was eluted from heparin agarose at higher salt concentrations (0.3 M-0.6M NaCl) than ATIII Random (0.3M). The heparin dissociation constants (K_D) were determined to be 3.5 x 10^{-7}M for ATIII(123-139) and 5.4 x 10^{-7}M for ATIII Random. Both peptides had binding constants within an order of magnitude of the native human protein. The heparin K_D for human ATIII was determined by the same assay to be 1 x 10^{-7} M. Using the same assay, a peptide from a putative heparin binding sequence

of the basic fibroblast growth factor, was found to have a K_d in the millimolar concentration range. It is interesting to note that the binding constants of ATIII(123-139) and ATIII Random differ very little, while their conformations in the presence of heparin is strikingly different.

Using a preparation of heparin with high affinity for human ATIII, it was demonstrated that the peptide ATIII(123-139) does not experience the same increase in affinity as human ATIII. This last finding, is a strong indication that the peptide cannot recognize the same pentasaccharide binding that is recognized by ATIII. A further experiment would have been to isolate enough high affinity heparin to show that the structure of the peptide ATIII(123-139) is the same with either crude or high affinity heparin.

In all the experiments performed with the glycopeptide, ATIII N135 GlcNAc behaved virtually identically to ATIII(123-139).

Chapter 5 References

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Ch. 5 Experimental

Materials and Supplies

Heparin-Agarose was purchased from Sigma (H-5380). A 50 ml. gradient mixer was borrowed from the laboratory of Professor Joanne Stubbe. ^{3}H-Heparin was purchased from DuPont-New England Nuclear. Nitrocellulose filters, 2.5 cm diameter, were purchased from Millipore. Bovine Serum Albumin was purchased from Sigma. Human Antithrombin III-Agarose was purchased from Sigma (A-8293). Human Anithrombin III was isolated by Cutter Pharmaceutical and provided as a generous gift from Professor Robert Rosenberg. Pico-Fluor 40 Scintillation cocktail was purchased from Packard.

Circular Dichroism Spectroscopy

See Chapter 4 Experimentals

General procedure for Heparin-Agarose Affinity Chromatography

A 5 x 1 cm column of Heparin Agarose (0.75 mgs heparin/ml.) was poured in a 10 x 1 cm Econopak column and outfitted with an Econopak flow adaptor. The column was initially equilibrated with 5 column volumes of Buffer A (50 mM Tris, pH 7.5), and subsequently hooked up to a fraction collector. 0.3 umoles peptide was diluted in 10mls of Buffer A (0.03 mM). The peptide sample was eluted on to the column at ~ 0.2 mls./min, followed by 5 mls of Buffer A at 0.2 ml./min. An 80 ml linear gradient was run from Buffer A to Buffer B (50mM Tris, 1 M NaCl (or salt of choice), pH 7.5). The column was washed with 5 mls. Buffer B. 48, 80 drop fractions were collected. The fractions were read for Absorbance at 276 nm on a Hewlett-Packard 8452Ato detect the peptide. The fractions were read with a conductivity meter to verify the salt gradient.

General Procedure for the Filter Binding Assay

Materials:

1.Nitrocellulose Filters (Millipore VCWP-02500, 0.10 uM poresize, 25 mm)

2. 1 mCi ^{3}H-Heparin (New England Nuclear, NET-476 Heparin Sodiumn Salt)

3. Bovine Serum Albumin (Sigma-A-9647)

4. Rinsing Buffer: 10 mM Tris, 150 mM NaCl, pH 8.0

5. Binding Buffer: Rinsing Buffer + 0.4% BSA

6. Filter Binding Apparatus hooked up to an aspirator.

Procedure:

1 mCi of ^{3}H-Heparin was dissolved in H_2O to give 1 mCi/ml. stock solution. This stock was diluted further, in series, to a final concentration of 0.1 uCi/ml. A 3.6 uM solution of human ATIII was prepared in binding buffer. Nitrocellulose filters equilibrated in the rinsing buffer ~ 1 hour before use.

The following series of samples (total volume 500 uls) was prepared in duplicate.

ATIII	Heparin	Binding Buffer
0	10 uls	490 uls
2 uls	10 uls	488 uls
5 uls	10 uls	485 uls
10 uls	10 uls	480 uls
20 uls	10 uls	460 uls
35 uls	10 uls	455 uls
50 uls	10 uls	440 uls
100 uls	10 uls	390 uls
200 uls	10 uls	290 uls
400 uls	10 uls	90 uls

The sample equilibrate at romm temperature for 45-50 minutes before application to the nitrocellulose filters. Each sample was applied slowly to the filter. The filter is slowly rinsed with 2 mls. of rinsing buffer. A pencil was used to gently mark each filter. The filters are dried for - 4 hours at 40 °C. Each filter is placed sample side up in the the bottom of a 20 ml. glass scintilation vial. 5 mls. of Pico-Fluor 40 scintiallation cocktail was added to each vial. The samples were counted for 1 minute each with luminescence

detection. Better readings were optained if the filters were allowed to sit in the scintilation cocktail for ~ 8 hours before counting.

Isolation of "High-Affinity "Heparin:

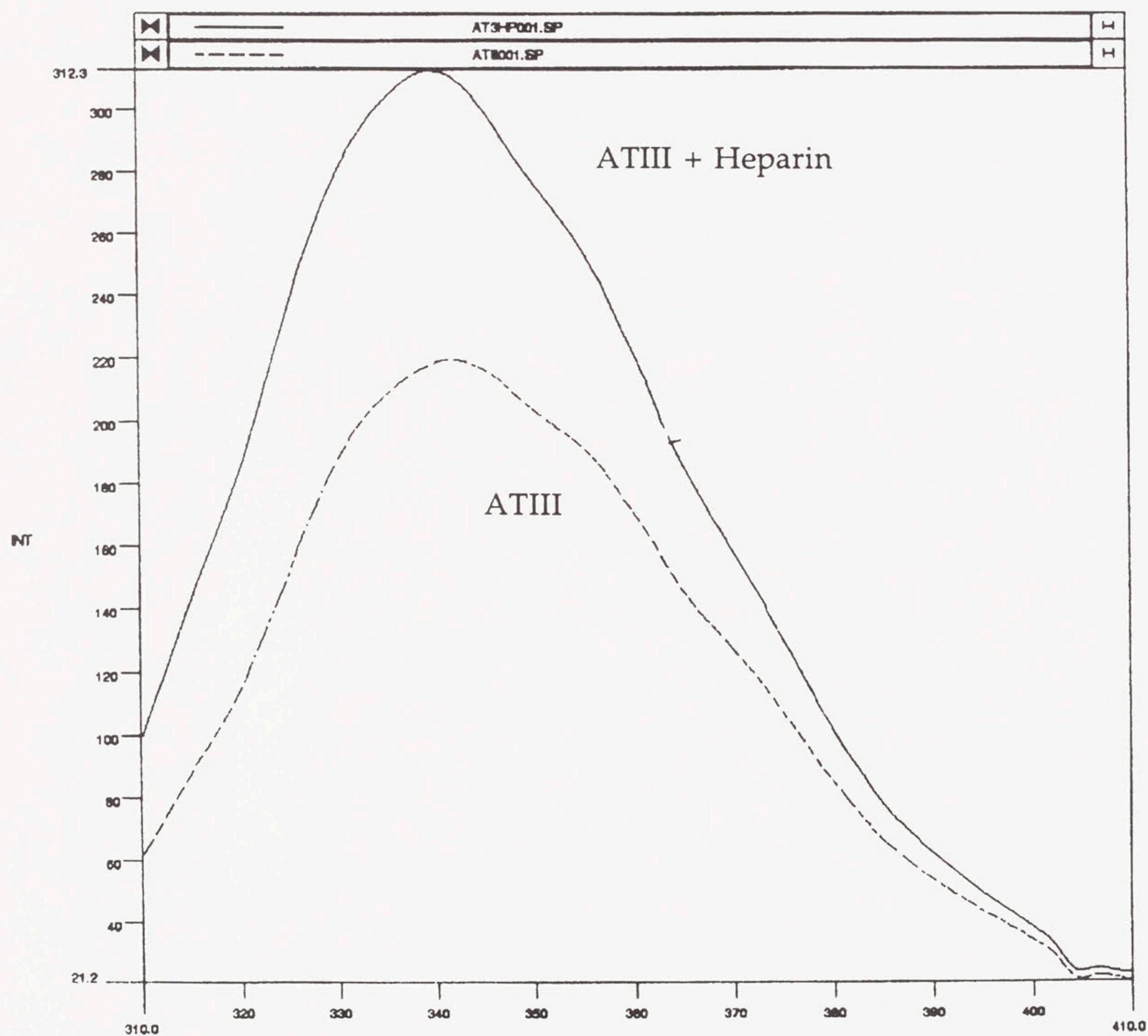
The following procedure is based on an experiment described in Lindhardt et al. J. Biol. Chem. (1992), 267, 2380-2387.

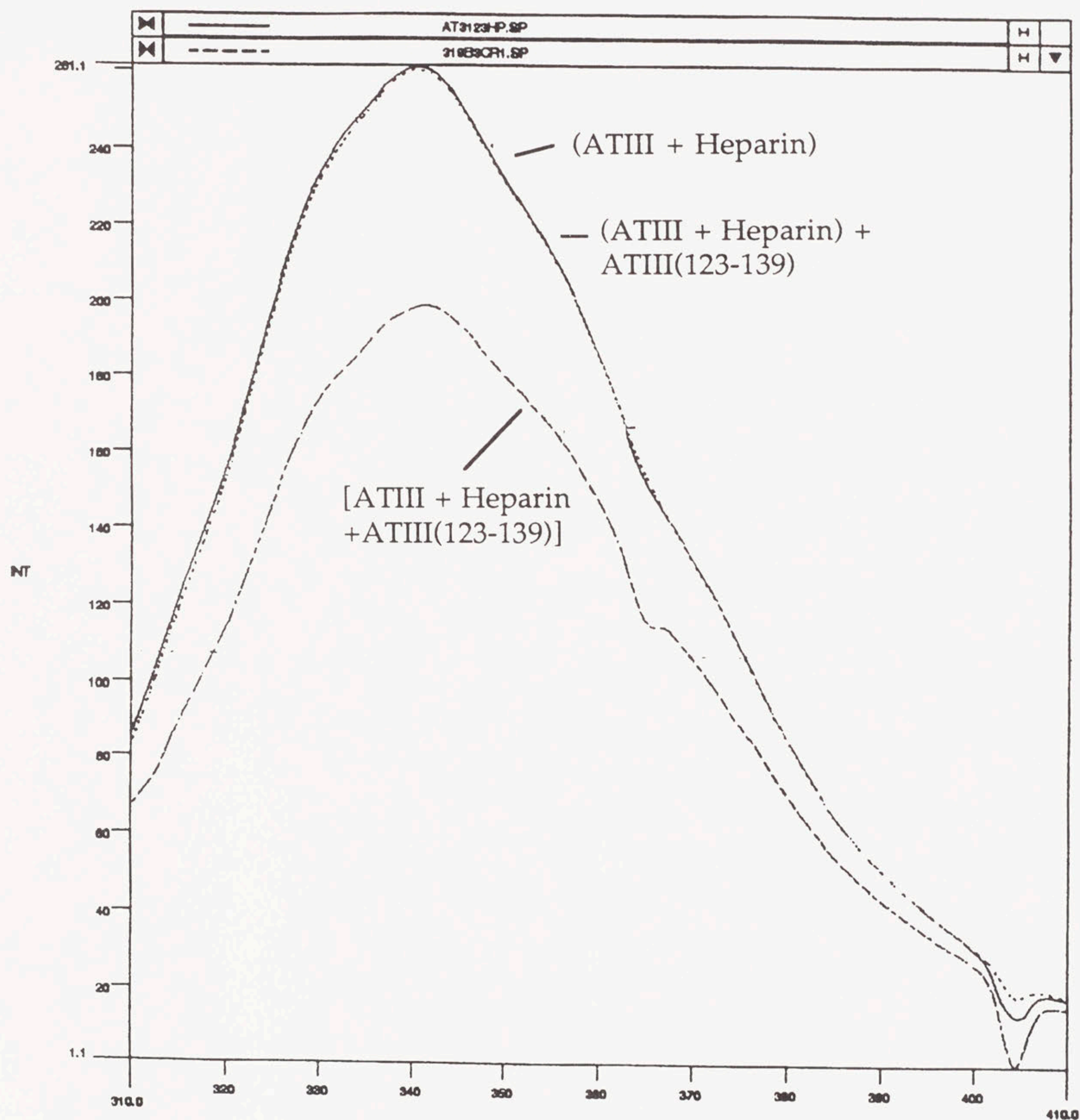
150 mgs. of dry ATIII-agarose swelled overnight in 6 mls. cold filtered 20 mM Tris, 0.05M NaCl, pH 7.5. 100 uls of the swelled resin was placed in an Econopak column and equilibrated in 20 mM Tris, 0.05 M NaCl, pH 7.5. 100 ul of a 1mCi/ml (2.3 mg/ml, 230 ug.) ^{3}H-Heparin stock solution in H_2O was added to 40 mM Tris, 0.10 M NaCl, pH 7.5. The ^{3}H-heparin sample was added to the ATIII-column. The following cycle of washes was performed and the eluent collected in 1 ml aliquots. 1) 10 mls 20 mM Tris, 0.05 M NaCl, pH 7.5. 2) 5 mls. 20 mM Tris, 0.25 M NaCl, pH 7.5. 3) 5 mls. 20 mM Tris, 1.0 M NaCl, pH 7.5. 20 uls of each aliquot of eluent was added to 5 ls. pico-Fluor 40 scintillation cocktail and counted for 1 min each. The first two fractions of the 1.0 M NaCl wash were collected as the high affinity heparin. The high affinity heparin was desalted on a Centricon 3 (3000 MW cut-off). After the initial 2 ml. fraction volume was reduece d to ~ 250 uls., the sample was spun down 3x out of 2 mls. Milli-Q H_2O. 10 uls of each rinse (flow through) was counted for 1 min. The final sample was counted and diluted to match the crude heparin stock solutions used in the filter -binding assay (10 ul stock solution + 5 mls. Pico-Fluor 40 = 2000 CPM). The heparin isolated here as "high affinity" heparin shows the expected higher binding constant with human Antithrombin III.

Fluorescence spectroscopy:

Fluorescence spectroscopy was performed on a Perkin Elmer LS 50 in the laboratory of Dr. Paul Schimmel, Department of Biology, MIT. The sample cell was purchased from

Hellma. All samples were excited at 300 nm with a 2.5 nm bandwidth. Emission spectra were scanned 4x from 310 nm to 410 nm at a 5.0 nm band width. Readings were obtained at 340 nm and integrated over 20 seconds. All sample readings were corrected for any solvent contributions.




AT3123HP.SP
(ATIII + Heparin)
(ATIII + Heparin) + ATIII(123-139)
[ATIII + Heparin +ATIII(123-139)]
INT
1.1
20
40
60
80
100
120
140
240
310.0
320
330
340
350
370
400
410.0