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SPECTROSCOPIC AND FUNCTIONAL STUDIES OF BOVINE MEIZOTHROMBIN(DESFL), HUMAN α-THROMBIN, AND HUMAN TISSUE PLASMINOGEN ACTIVATOR

Dissertation

Presented in partial fulfillment of the Requirements for the Degree Doctor of Philosophy in Graduate School of The Ohio State University

By

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*****

The Ohio State University

1996

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FIELD OF STUDY

Major Field:
Chemistry
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<tr>
<td>6FT</td>
<td>6-fluorotryptamine</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>a</td>
<td>molecular radius</td>
</tr>
<tr>
<td>A</td>
<td>hyperfine splitting constant; absorbance</td>
</tr>
<tr>
<td>A&lt;sub&gt;em&lt;/sub&gt;</td>
<td>absorbance at emission wavelength</td>
</tr>
<tr>
<td>A&lt;sub&gt;exc&lt;/sub&gt;</td>
<td>absorbance at exciting wavelength</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>APC</td>
<td>activated protein C</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β</td>
<td>Bohr magneton</td>
</tr>
<tr>
<td>bis-ANS</td>
<td>4,4'-bis 1-(phenylamino)-8-napthalenesulfonate</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade (temperature)</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>chromothrombin</td>
<td>tosyl-L-glucyl-L-arginyl-p-nitroanilide</td>
</tr>
<tr>
<td>chromogenic TH</td>
<td>ethylmalonyl-thioproyl-L-arginyln-p-nitroanilide</td>
</tr>
<tr>
<td>Dansyl-EGR-CH$_2$Cl</td>
<td>dansyl-L-glutamyl-L-glucyl-L-arginyl-chloromethyl-ketone</td>
</tr>
<tr>
<td>DAPA</td>
<td>dansylarginine N-(3-ethyl-1, 5-pentanediyl)amide</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethyliaminoethyl</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DOPC</td>
<td>dioleyl-phosphatidylcholine</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>e</td>
<td>extinction coefficient</td>
</tr>
<tr>
<td>e-ACA</td>
<td>e-amino caproic acid</td>
</tr>
<tr>
<td>E</td>
<td>energy</td>
</tr>
<tr>
<td>e</td>
<td>electron charge</td>
</tr>
<tr>
<td>ECV</td>
<td><em>Echis carinatus</em> venom</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-N, N, N', N'-tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>F</td>
<td>fluorescence intensity</td>
</tr>
<tr>
<td>F$_0$</td>
<td>emission intensity in the absence of quencher</td>
</tr>
<tr>
<td>FI</td>
<td>prothrombin fragment 1 (residues 1-151)</td>
</tr>
</tbody>
</table>
F2  prothrombin fragment 2 (residues 152-274)
g  Landé factor describing the total momentum of an electron; gram
Glu  glutamic acid
Gly  glycine
η  viscosity
H  magnetic field
h  Plank’s constant
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His  histidine
Iz  nuclear spin
Ile  isoleucine
k  Boltzmann constant; rate constant describing the decay of $S_1$
ket  rate constant describing energy transfer
kr  rate constant of fluorescent radiative decay
ki  rate constant describing internal conversion
kq  rate constant of collisional quenching
ks  rate constant describing intersystem crossing
Kd  dissociation constant
Ksv  Stern-Volmer quenching constant
l  path length
Leu  leucine
μ  magnetic moment
M  molar
m  mass
m-IV  (m-CO-6NH) 3-m-(fluorosulfonyl)benzamide
m-V  (m-CO-5NH) 3-(2,2,5,5-tetramethyl-pyrrolidine-1-oxyl)-m-(fluorosulfonyl)benzamide
m-VII  (m-NCO-6NH) N[m-(fluorosulfonyl)phenyl]-4-N-(2,2,6,6-tetramethyl-piperidine-1-oxyl) urea
mg  milligrams
min  minutes
mL  milliliters
mOD  milli- optical density units
NPGB  p-nitrophenyl-p-4'-guanidinobenzoate
OD  optical density
P  fraction of population 1 contributing to fluorescence quenching
p-I  (p-NH-5=CO) N-(4-(fluorosulfonylphenyl)-2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxamide
p-III  (p-CO-6OH) 3-(2,2,5,5-tetramethyl-pyrrolidine-1-oxyl)-p-(fluorosulfonyl)benzoate
p-IV  
*(p-CO-6NH)* 4-(2,2,6,6-tetramethyl-piperidine-1-oxyl)-p-(fluorosulfonyl)benzamide

p-V  
*(p-CO-5NH)* 4-(2,2,5,5-tetramethyl-pyrrolidine-1-oxyl)-p-(fluorosulfonyl)benzamide

PEG6000  Polyethylene glycol (ave. M.W. 6000)

pH  
-log₁₀ [Hydrogen ion concentration]

Phe  phenylalanine

PPACK  d-phenylalanyl-L-argininyl-chloromethyl ketone

q  quantum yield in the presence of quencher

q₀  quantum yield in the absence of quencher

[Q]  molar concentration of quencher

R  universal gas constant

rpep  thrombin receptor peptide

s  seconds

S₀  ground electronic state

S₁  first excited electronic state

S-2160  benzyl-L-phenylalanyl-L-valinyl-L-arginyl-p-nitroanilide

S-2238  h-D-phenylalanyl-piperidinyl-L-arginyl-p-nitroanilide

S-2288  h-D-isoluecyl-L-prolyl-L-arginine-p-nitroanilide

S-2366  L-glutamyl-L-prolyl-L-arginyl-p-nitroanilide

sc-tPA  single chain tissue plasminogen activator

SDS-PAGE  sodium dodecylsulfate polyacrylamide gel electrophoresis

SL3  (DL)-3-carboxy 2,2,5,5-tetramethyl pyrrolidine-N-oxyl-p-nitrophenyl ester

ᵦᵣ  rotational correlation time

t  time

T  absolute temperature

TAME  tosyl-arginine methyl ester

tct-PA  two chain tissue plasminogen activator

TM  thrombomodulin

TMD1(CS+)  recombinant thrombomodulin containing chondroitin sulfate

Tris  Tris-(hydroxymethyl)amino methane

Trp  tryptophan

Tyr  tyrosine

υ  frequency

UV  ultraviolet

V  partial specific volume

Val  valine

vs.  versus
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INTRODUCTION

1.1 OVERVIEW AND RESULTS

Activation of prothrombin by the prothrombinase complex results in two active intermediates meizothrombin (mzIIa) and meizothrombin(desF1) (mzIIa-F1), and ultimately α-thrombin (Heldebrant et al., 1973; Rosing and Tans, 1988; Krishnaswamy et al., 1986; Bovill et al., 1995). Both mzIIa and mzIIa-F1 possess full small substrate activity, while mzIIa exhibits < 1 % clotting activity of α-thrombin (Doyle and Mann, 1990). MzIIa-F1 shows limited clotting activity (10 % of α-thrombin), while both show significant activity toward protein C in the presence of thrombomodulin, an integral membrane protein found in platelets which upon binding to α-thrombin, converts it to an anticoagulant (Doyle and Mann, 1990; Esmon, 1993). To date, no X-ray crystal structure of either of these important intermediates has been published.

The active site of bovine mzIIa-F1 was probed by the use of several sulfonyl fluoride spin labels, which orient toward different regions of the active site (Berliner and Wong, 1974). Large differences between bovine α-thrombin and mzIIa-F1 in the mobilities (measured by 2T₁) of the labels which are believed to probe the indole binding region of the active site were found (Bode et al., 1992). One label, p-V was found by crystallography to
reside in the fibrinogen binding groove (subsites S2-S3; Nienaber and Berliner, 1996). It is presumed that other labels of similar orientation (p-I, p-III, and p-IV) probe a similar region of the active site. MzIIa-F1 was also found to possess an indole binding site as spectra of spin labeled mzIIa-F1 showed changes in the presence of 20 mM indole. However, no activation of TAME esterase activity was noted for mzIIa-F1. In the other class of labels studied (those in which the sulfonyl fluoride moiety is in the meta position), little differences were noted. Thus, the region these labels probe (found by crystallography to be the S1 specificity pocket and autolysis loop) is of similar conformation in mzIIa-F1 (Bode et al., 1992; Nienaber and Berliner, 1996). Studies utilizing the inhibitor benzamidine revealed that both meta and para labels of α-thrombin and mzIIa-F1 experienced an immobilization upon the addition of benzamidine, with a K_D app of 1.0 ± 0.1 mM for both labeled enzymes. Benzamidine concentrations of up to 100 mM had little or no effect on the p-I, and p-V labeled enzymes. Indole of a concentration of 20 mM was found to influence mzIIa-F1 label mobility as has been found with α-thrombin. Unlike with α-thrombin, however, p-V exhibited no change in mobility, while a dramatic immobilization was noted for m-VII labeled mzIIa-F1, compared with a modest increase for α-thrombin. Activity studies also showed that mzIIa-F1 experiences no indole-induced activation of esterase activity in contrast to the 3-fold activation seen with α-thrombin. All of the above data point toward an "immature" indole site in mzIIa-F1 which does not initiate the same conformational changes as it does in α-thrombin.

α-Thrombin exists under physiological conditions as two conformations, termed fast and slow by Wells and Di Cera (1992) since the fast form exhibits more efficient cleavage
of most substrates. However, the slow form does cleave protein C more effectively, making this enzyme an anticoagulant (Dang et al., 1995). The slow $\rightarrow$ fast conversion is facilitated by the binding of a Na$^+$ ion to a site located near the base of the S1 specificity pocket (Di Cera et al., 1995). The conformational changes that $\alpha$-thrombin undergoes during this conversion have been examined in the past by Villanueva and Perret (1983), who concluded from circular dichroism (CD) studies that tyrosine residues are involved in the transition. Fluorescence studies in this work show that the surface of slow $\alpha$-thrombin is less hydrophobic than that of fast $\alpha$-thrombin, having obvious implications on the binding of apolar ligands. Guanidine denaturation studies demonstrate that slow $\alpha$-thrombin is more stable by approximately 0.5 M guanidine. However, both conformations were found to be equally stable in thermal denaturation studies with melting temperatures of 53°C. Quenching studies of slow and fast $\alpha$-thrombin suggest that tryptophans are not involved in the conversion because no change in tryptophan exposure to solvent was noted. Fluorescence of dansyl and dansyl-EGR- $\alpha$-thrombin are consistent with a constricting of the fibrinogen binding groove in slow $\alpha$-thrombin.

Inhibition of slow and fast $\alpha$-thrombin with proflavin, tryptamine, and 6-fluorotryptamine (6FT) reveals some interesting results as well. Under fast conditions, these are simple competitive inhibitors of $\alpha$-thrombin activity (Casale et al., 1995). However, under slow conditions, this is no longer the case. Inhibition is indicative of at least two binding sites. Under conditions of saturating substrate, activation is observed at low concentrations of 6FT, followed by inhibition at higher concentrations. Binding of 6FT to the S1 specificity binding pocket is disrupted in the absence of Na$^+$ (slow conditions).
Activation of α-thrombin activity is thought to be due to an increase in the deacylation rate upon indole binding (i.e. indole and indole derivatives destabilize the acylated enzyme). Since, as it is with α-thrombin esterase activity, the deacylation step is rate limiting for amidolytic cleavage by slow α-thrombin (Wells and Di Cera, 1992), the presence of indole derivatives induce an increase in α-thrombin activity under these conditions. Fast α-thrombin exhibits no activation since acylation is rate limiting. In the case of proflavin inhibition, clearly binding to S1 is disrupted. However, no activation is observed under slow conditions.

In contrast to most serine proteases, the zymogen, single chain form, of human tissue plasminogen activator (t-PA) possesses significant activity (17 - 30 % of two chain; Tate et al., 1987; Boose et al., 1989). This is possibly due to the flexibility of the one chain form (sct-PA) to convert to the two chain conformation (tct-PA) without the cleavage necessary for the production of tct-PA. This hypothesis was tested by the utilization of a spin label (SL3) which forms a trigonal complex with t-PA. Tetrahedral spin labels have shown no differences between the two forms (Nienaber et al., 1992). A small, but significant, decrease in spin label mobility was observed upon conversion of SL3 labeled sct-PA to tct-PA. This decrease was also exhibited upon the addition of 100 mM ε-amino caproic acid, mimicking the activation by fibrin on the lysine binding site of t-PA. Tct-PA did not show this change. Thus, this data supports the hypothesis that sct-PA exists in a "slow" conformation, but can be converted to a "fast" conformation by the occupation of the lysine binding of t-PA or by the presence of a substrate (with a tetrahedral intermediate) in the active site.
1.2 SYSTEM UNDER INVESTIGATION

1.2.1 The clotting pathway.

\( \alpha \)-Thrombin is a serine protease which is central to blood coagulation. The major function of \( \alpha \)-thrombin is the cleavage of soluble fibrinogen to insoluble fibrin, by the removal of two fragments, fibrinopeptides A and B. \( \alpha \)-Thrombin's role in the blood clotting pathway is diagrammed in Figure 1.1. The clotting cascade consists of two pathways, extrinsic and intrinsic. Current thought is that the extrinsic pathway, which is initiated by vascular damage, is critical to the formation of fibrin, while the intrinsic pathway, which is activated by \( \alpha \)-thrombin, is necessary for the maintenance of fibrin once it is formed (Davie et al., 1991). The extrinsic pathway, therefore, is responsible for the majority of the \( \alpha \)-thrombin formed, and occurs on a shorter time scale.

Activation of the extrinsic pathway is initiated by the release of tissue factor from damaged endothelial cells (tissue factor is normally present tightly bound to phospholipids in the membrane). Released tissue factor then forms a tight complex with factor VII and results in the proteolytic formation of factor VIIa by catalytic levels of a circulating protease (Nemerson and Repke, 1985). The newly formed tissue factor - factor VII complex is now able to cleave factor IX into factor IXa, which in turn activates factor X to Xa. Factor Xa coupled with factor Va (present in very low quantities until \( \alpha \)-thrombin catalyzes the cleavage of factor V to Va), negatively charged phospholipids, and calcium ions form the prothrombinase complex, which is responsible for the conversion of prothrombin to \( \alpha \)-thrombin (Mann et al., 1991; Krishnaswamy, 1990). The intrinsic pathway is somewhat more complex. \( \alpha \)-Thrombin on a negatively charged surface will cleave factor XI to XIa.
The clotting cascade can be divided into two pathways, intrinsic and extrinsic. The extrinsic pathway is initiated by release of tissue factor and is responsible for the majority of α-thrombin produced during clotting. The intrinsic pathway is initiated by α-thrombin and plays a role in clot maintenance. The heavy arrows refer to those pathways which produce a large amount of product. Taken from Davie et al. (1991).
Figure 1.2: A schematic of the possible pathways of prothrombin activation. Prothrombin consists of four domains. Fragments 1 and 2, represented by F1 and F2, respectively, are cleaved off during the conversion of prothrombin to α-thrombin. The resulting A and B chains ultimately make up the α-thrombin molecule. Meizothrombin, meizothrombin(desF1), and α-thrombin all exhibit full activity toward small substrates. However, only α-thrombin possesses full clotting activity, while mzIIa-F1 exhibits limited clotting activity (<10% of α-thrombin) and mzIIa shows virtually none (<10%; Doyle and Mann, 1990). Because bovine meizothrombin is quite susceptible to autodegradation resulting in stable meizothrombin(desF1), the latter is the subject of study in this work.
Factor XIa, in the presence of calcium ions, activates factor IX to IXa, which in turn activates factor X to Xa, resulting in formation of the prothrombinase complex and the production of α-thrombin. It is this pathway which causes blood which is stored in glass vessels to clot, since glass provides a negatively-charged surface. The conversion of prothrombin to α-thrombin has been intensely studied and will be considered in greater detail. The structures of prothrombin and some activation intermediates are illustrated in Figure 1.2. Prothrombin consists of four domains. Residues 1-155 constitute fragment 1, which is necessary for binding to negatively charged lipid surfaces. Residues 156-271 make up fragment 2, which probably is responsible for interactions with other proteins such as factor Va. Finally, α-thrombin itself is residues 272-589, with residues 272-330 being the disulfide linked A-chain. In order for α-thrombin to be formed from prothrombin, at least two cleavages must occur. One cleavage, at Arg271 (human prothrombin numbering), is necessary for the removal of fragments 1 and 2. The second cleavage occurs at Arg330. It is this cleavage which results in the formation of the fully functional active site (Doyle and Mann, 1990). In the presence of the entire prothrombinase complex, the formation of α-thrombin occurs via an ordered sequential reaction with the Arg330 occurring first (Krishnaswamy et al., 1986; Rosing et al., 1986; Rosing and Tans, 1988). The intermediate which is formed in this reaction, meizothrombin (mzIIa), possesses a fully functional active site, but the prothrombin fragments are still covalently attached. Despite the fact that mzIIa retains activity for small tripeptide substrates, it exhibits very little clotting activity (< 1% that of α-thrombin). It does, however, activate the anticoagulant pathway through the binding to thrombomodulin (TM) and subsequent cleavage of protein C to activated protein C (APC), an inactivator of
factor Va (Doyle and Mann, 1990).

Study of mzIIa is complicated by the fact that mzIIa is not stable in solution. MzIIa will quickly autocatalyze the cleavage of fragment I at Arg155, resulting in meizothrombin(desF1) (mzIIa-F1). In the human form, mzIIa-F1 is also unstable and quickly converts to α-thrombin through autocatalysis as well. However, the bovine form is quite stable and is used as the source of all of the mzIIa-F1 studies in this work. MzIIa and human mzIIa-F1 can be studied through the use of strongly binding reversible inhibitors (Doyle and Mann, 1990) or site directed mutagenesis (Pei et al., 1992; Côté et al., 1994).

Once the tissue around the vascular injury has healed sufficiently, the clot becomes the target of fibrinolysis. The dissolution of the clot into soluble fragments is accomplished by proteolytic degradation of the fibrin by plasmin. The activation of plasmin from plasminogen is effected in vivo by the serine proteases tissue plasminogen activator (t-PA), urokinase (u-PA), and kallikrein activated plasminogen activator (Higgins and Bennett, 1990). Of these, only t-PA will be discussed in this work. T-PA is secreted into the bloodstream by endothelial cells and removed from circulation by PAI-1 (a serine protease inhibitor) as well as clearance by the liver. Typical of serine proteases, t-PA circulates in the plasma as the zymogen one-chain form (sct-PA) until subsequent activation by plasmin. T-PA is atypical, however, in that its zymogen form possesses significant activity (17 - 33 % of the activated form; Tate et al., 1987; Boose et al., 1989).

1.2.2 The structural components of α-thrombin.

The crystal structure of α-thrombin, shown in Figure 1.3A, was solved in 1989 by
Bode et al. (1989) and was found to be highly homologous to chymotrypsin, with several insertion loops (Bode et al., 1992). A cartoon schematic of this structure is presented in Figure 1.3B, which highlights several important \( \alpha \)-thrombin regulatory sites. The specificity of \( \alpha \)-thrombin towards its substrates is determined by several aspects of its structure. In this work, the subsites of the enzyme active site which bind the substrate are identified as S1, S2, S3, etc. numbering from the scissile bond toward the N-terminus of the peptide. Subsites on the C-terminal side are labeled S1', S2', S3', etc. Similarly, the residues of the substrate are labeled P1, P2, P3, etc. and P1', P2', P3', etc. (for a discussion of this nomenclature see Fersht, 1985). At the base of the primary specificity pocket S1 is Asp\(^{189} \) (designated "R" in Figure 1.3B), which results in the specificity for long positively charged side chains (Lys and Arg) at the P1 position of the substrate. Apolar residues in subsites S2 and S3 also produce an affinity for small aliphatic residues in P2 (Pro, Gly, and Val) and aromatic residues in P3 (De Nanteuil et al., 1995). Specificity is also affected by a large patch of positively charged residues (designated "F" in Figure 1.3B) termed anion exosite I. This site is necessary for fibrinogen recognition as it binds a series of acidic residues in fibrinogen (Jakubowski and Owen, 1989; Bode et al., 1992). There are also several insertion loops which are of importance. Residues 60A-60H (chymotrypsinogen numbering) form a rigid loop covering edge side of the active site groove. The other edge, a more flexible loop, consists of residues 144-150. Both of these loops are important in determining the macromolecular specificity of \( \alpha \)-thrombin through steric hindrance of the active site groove. A third insertion loop exists at residue 221. This makes up the Na\(^{+} \) binding site and will be discussed in the next section.
1.2.3 Regulation of α-thrombin.

In order for haemostasis to be successful, α-thrombin production and activity must be delicately controlled. Once a clot is formed, α-thrombin must be inactivated quickly to prevent unwanted clotting, which could easily mean illness or death for the organism. This removal of α-thrombin is accomplished in vivo by an anticoagulation pathway. α-Thrombin binds the integral membrane protein thrombomodulin (TM; present in the platelet membrane). TM binds to α-thrombin in the anion exosite I (described in section 1.2.2) and converts α-thrombin to an anticoagulant enzyme, by a mechanism which is yet to be fully elucidated (Ye et al., 1992; Esmon, 1993). This α-thrombin-TM complex is able to cleave protein C much more efficiently into activated protein C (APC; Esmon, 1993). Factor Va is then inactivated by APC, thus preventing the formation of more α-thrombin (see Figure 1.1). Free α-thrombin is inactivated by an irreversible inhibitor antithrombin III (ATIII) in the presence of heparin, a sulfated polysaccharide which acts as a bridge between α-thrombin and ATIII. The inactivated enzyme is then removed from the plasma by general proteases.

The activity of α-thrombin is also allosterically regulated during the clotting process. One allosteric ligand is Na⁺. Work in Dr. E. Di Cera's laboratory has shown that Na⁺ binds to α-thrombin specifically and converts it from a "slow" form to a "fast" form (Wells and Di Cera, 1992; Dang et al., 1995; Di Cera et al., 1995). The slow/fast nomenclature arises from the relative abilities of the two forms to cleave fibrinogen and other substrates to fibrin. The slow form of α-thrombin cleaves fibrinogen less efficiently than fast α-thrombin, but is
Figure 1.3: The crystal structure of α-thrombin. A. Ribbon diagram of the crystal structure of α-thrombin solved by Bode et al. (1989). B. A cartoon schematic of the important sites of α-thrombin. Subsites of the α-thrombin active site are designated S1 through S6 and S1' through S4'. H and F refer to anion exosites I and II, respectively. A and C designate the indole and aryl sites, respectively. G and R refer to important surface residues. R is Asp\textsuperscript{189} at the base of the S1 specificity pocket, while Glu\textsuperscript{39} represents an important charge interaction with TM. Figures 1.3A and 1.3B were taken from Bode and Stubbs (1993) and Stubbs and Bode (1993), respectively.
Figure 1.3.
able to cleave protein C at a higher rate, acting as an anticoagulant. The dissociation constant \( (K_D) \) for Na\(^+\) binding at 25°C is 24 mM, well below the physiological concentration of Na\(^+\). However, the binding of Na\(^+\) has a very large enthalpy component and the dissociation constant is quite temperature dependent. Under physiological conditions, the relative population of slow \( \alpha \)-thrombin is 39% (Dang \textit{et al.}, 1995). Thus, Na\(^+\) acts as a passive activator by maintaining approximately equal concentrations of slow and fast \( \alpha \)-thrombins. Any phenomenon which shifts this equilibrium has the effect of activation or inactivation depending on the direction of the equilibrium shift (Lord \textit{et al.}, 1995; De Cristofaro \textit{et al.}, 1995). This is illustrated in Figure 1.4. An example of such an effector is TM, which binds preferentially to the fast form, thus acting as an anticoagulant.

The binding site of Na\(^+\) has been determined by mutagenesis and crystallographic methods to be the backbone carbonyl oxygens of Tyr\(^{184A}\), Arg\(^{221A}\), and Lys\(^{224}\) (Di Cera \textit{et al.}, 1995). The Na\(^+\) site and its relationship to the rest of the \( \alpha \)-thrombin molecule is presented in Figure 1.5. The general location of this site is very near the base of the S1 pocket, in a nearby solvent channel. The large enthalpy change observed in the binding of Na\(^+\) is most likely due to rearrangement of water hydrogen bonding networks in this channel (De Cristofaro \textit{et al.}, 1995; Di Cera, 1995). The conformational changes which occur during conversion are not known to any great detail.

1.2.4 Apolar binding sites of \( \alpha \)-thrombin.

Another avenue of thrombin regulation occurs through the release of tryptophan derivatives by activated platelets, principally serotonin, tryptamine, and ATP. The structures
Figure 1.4: Schematic of α-thrombin regulation by Na⁺.

α-Thrombin exists, under physiological conditions, at equilibrium between slow and fast forms. Ligands which shift this equilibrium act as anticoagulants or coagulants depending on the direction of equilibrium shift. Taken from Dang et al. (1995).
Figure 1.5: The Na⁺ binding site as determined crystallographically by rubidium replacement. A. The binding site of Na⁺ as it relates to the α-thrombin molecule. In this figure 1, 2, 3, and 4 refer to the Na⁺ binding loop, autolysis (or γ-) loop, anion exosite I, and anion exosite II, respectively. B. The X-ray structure of the coordination of the Na⁺ ion.

The Na⁺ site is located in a solvent channel near the base of the S1 specificity pocket. It is coordinated by the backbone carbonyl oxygens of Tyr184A, Arg221A, and Lys234, as well as three water molecules. Taken from Di Cera et al. (1995).
of these molecules and some of their derivatives are given in Figure 1.6. The effects of these apolar molecules on α-thrombin have been studied in the past by Berliner and coworkers (Berliner and Shen, 1974; Conery and Berliner, 1983; Berliner et al., 1986) and have been found to be quite complex. A good deal of evidence was found that supports the idea that most of these may bind α-thrombin in two sites (Berliner et al., 1986). An example of the effect of apolar ligands on α-thrombin activity is shown in Figure 1.7. Clotting activity was found to be activated at low concentrations of apolar ligands (2 mM), followed by inhibition at higher concentrations of ligand. This activation was observed for esterase activity as well, but not for amidolytic activity. Because of this, and the high dissociation constants measured, this work has been criticized for being "unphysiological." However, since these molecules are secreted in very high local concentrations and clear effects are observed on α-thrombin activity, they may play an important role in α-thrombin regulation.

Studies employing crystallography have been largely inconclusive in determining the binding sites of tryptophan derivatives. Upon the determination of the α-thrombin crystal structure, the apolar site was proposed to be a cluster of hydrophobic residues near and in the active site as shown in Figure 1.8 (Stubbs and Bode, 1993). This site, however, was inconsistent with activation of clotting activity. Attempts to co-crystallize α-thrombin with apolar ligands have been unsuccessful. Recently, Nienaber and Berliner (1996) have soaked α-thrombin crystals with 6-fluorotryptamine (6FT) and this structure is discussed in chapter IV. The ligand was found to reside in the S1 pocket and most represents the inhibition site of 6FT. A second activation site was not found, but factors such as crystal packing may play a role in this lack of binding. The location of this proposed second site remains a mystery.
Figure 1.6: The structures of several molecules (and their derivatives) released by platelets during clotting.
Figure 1.7: The effect of several apolar ligands on clotting activity of \( \alpha \)-thrombin.

Ligands were found to activate to different degrees at low (1-3 mM) and inactivate at higher concentrations (>8 mM). A decrease in clotting time represents an increase in clotting activity. Taken from Berliner et al. (1986).
Figure 1.7.
Figure 1.8: A crystal structure of α-thrombin showing the apolar binding site of α-thrombin as proposed by Bode et al. (1992). This site consists of the following residues: His57, Tyr60A, Trp60D, Leu99, Ile174, and Trp215. The α-thrombin coordinates were obtained from the Brookhaven protein data bank (file 2hpq; Bode et al., 1989).
1.2.5 The structure of t-PA

The structural domains of tissue-type plasminogen activator (t-PA) are similar to those of several blood coagulation proteases. Shown in Figure 1.9, the secondary structure of t-PA consists of five domains: finger, epidermal growth factor (EGF), two kringles, and a serine protease domain (Higgins and Bennett, 1990). It is probable that all of the domains of t-PA are involved to a certain extent with interactions with fibrin (van Zonneveld et al., 1986; Kalyan et al., 1988; Higgins and Bennett, 1990). The finger domain and EGF domains have been implicated in binding fibrin, although the extent is somewhat controversial (van Zonneveld et al., 1986; Kalyan et al., 1988). Kringle domains are quite common among blood coagulation enzymes and are present on prothrombin, plasminogen, urokinase (u-PA), plasmin, and fibronectin (Tulinsky, 1991). They are generally involved in protein-protein interactions and are believed to play a part in t-PA interaction with fibrin. The presence of fibrin and fibrin degradation products induces a dramatic increase (up to a 1000-fold) in t-PA substrate activity (Bennett et al., 1991). Kringles 1 and 2 differ in that the latter possesses a lysine binding site (Wilhelm et al., 1990), which has been implicated in the activation of sct-PA by fibrin (Nienaber, 1990). The serine protease domain of t-PA is responsible for the cleavage of plasminogen, resulting in the formation of plasmin. The crystal structure of the catalytic (serine protease) domain of tct-PA has been recently reported by Lamba et al. (1996). The overall structure of the substrate cleft was found to be more sterically occluded and kinked than that of α-thrombin. One of the implications for the inducible activation of sct-PA is the stabilization of the Ile16 binding pocket with Asp194. In the activated two chain form, this pocket is occupied by the N-terminal Ile16.
1.3 BACKGROUND DESCRIPTION OF METHODS EMPLOYED

1.3.1 Electron Spin Resonance.

Electron spin resonance (ESR) spectroscopy is a highly specific technique which monitors only molecules with a paramagnetic moiety. In biological ESR this moiety can be a paramagnetic metal (in the study of metalloenzymes), a free radical intermediate (e.g. recent studies of \textit{in vivo} NO generation), or a stable radical used to probe the properties of a diamagnetic molecule (e.g. protein spin labeling). The ESR studies in this work are all of the latter type, in which a stable nitroxide is bound covalently to the active site of the enzyme of interest. The relative motion of this label under different experimental conditions is used to gain information about the system being studied.

1.3.1.1 Basic considerations in electron spin resonance studies.

The general structure of the paramagnetic moiety used in these studies is shown below. The unpaired electron is localized on the nitrogen - oxygen of the highly stable nitroxide. The R group
Figure 1.9 Structure of tissue plasminogen activator. The five domains shown are a finger, GF (or EGF), two kringle, and one serine protease domain. Figure taken from Verstraete and Collen (1986).
represents a structure which binds (covalently or non-covalently) to the molecule of interest, thus providing a means to probe the diamagnetic molecule by ESR.

In the absence of a magnetic field, the eigen values of an electron (+½ and -½) have energies which are degenerate. The energy levels can be described by the following equations:

\[ E = \mu H \quad (1.1) \]
\[ \mu = -g\beta s \quad (1.2) \]

where \( E \) is the energy of the state, \( \mu \) is the magnetic moment, \( H \) is the magnetic field, \( g \) is the electron g value, \( \beta \) is the Bohr magneton, and \( s \) is the electron spin quantum number. The g value for a free electron is 2.0023, while \( \beta \) is described by

\[ \beta = \frac{e\hbar}{4\pi mc} = 9.273 \times 10^{-19} \text{ erg} / \text{G} \quad (1.3) \]

where \( e \) is the electron charge \((4.80298 \times 10^{-10} \text{ esu})\), \( \hbar \) is Plank's constant \((6.6256 \times 10^{-27} \text{ erg s})\), \( m \) is the mass of an electron \((9.1091 \times 10^{-31} \text{ kg})\), and \( c \) is the speed of light \((2.997925 \times 10^8 \text{ m/s})\).

In the presence of a magnetic field, the spin states are longer degenerate with the -½ being of lower energy. The energy difference, termed the Zeeman effect, is described by

\[ \Delta E = \hbar \nu = g\beta H \quad (1.4) \]
and is illustrated in Figure 1.10. Thus, when the frequency is held constant and the magnetic field is scanned (as is done in a typical ESR experiment), an absorption of a photon of radiation occurs when the magnetic field satisfies equation 1.4. For nitroxides, these values are typically on the order of 9.5 GHz and 3300 G for the frequency and magnetic field, respectively.

1.3.1.2 The hyperfine interaction.

As in nuclear magnetic resonance (NMR), the ESR signal can be split by neighboring nuclei. In the case of nitroxides, interaction of the lone electron with the nitrogen nucleus (I=1) splits the ESR spectrum into three lines each corresponding to a nuclear spin state of the nitrogen nucleus (I_z = -1, 0, +1). The energy of transition previously described in equation 1.4 becomes

\[ \Delta E = g\beta H \pm \frac{1}{2}A m_1 \]

(1.5)

where A is the hyperfine splitting constant (½A is the hyperfine splitting energy) and m_1 is the value of I_z for the nitrogen nucleus. Thus, the ESR spectrum of a freely tumbling nitroxide in solution results in three symmetrical lines as shown in Figure 1.11.

1.3.1.3 Spectral Anisotropy.

Because the distribution of the lone electron about the nitrogen nucleus is not symmetrical, the contribution of the signal is dependent on the orientation of the molecule
**Figure 1.10:** The Zeeman effect on the energy levels of electron spin states.

As the strength of the magnetic field increases, the energy difference between the $+\frac{1}{2}$ and $-\frac{1}{2}$ spin states also increases. Taken from Knowles *et al.* (1976).
Figure 1.10.
towards the magnetic field. The nitroxide molecular axes are shown in Figure 1.12A. The corresponding ESR spectra of the contribution of each axis is shown in Figure 1.12B. Because these are different, the ESR spectrum is quite sensitive to the motion of the label. A freely tumbling nitroxide will result in three symmetrical lines because the different orientational contributions are averaged out by the motion of the molecule. However, if the motion of the label is slowed, all the orientations of the label begin to contribute to the spectrum. The effect of motion on the ESR spectrum of tempol, a common spin probe, is show in Figure 1.13. The majority of spectra of spin label bound to protein result in a spectrum which falls in the moderately to strongly immobilized category. The maximum hyperfine splitting ($2T_r$), which is the hyperfine splitting of the orientation parallel to the magnet, is determined as shown in Figure 1.14. This parameter can be used as a semi-quantitative measure of the mobility of the label (increases as label mobility decreases).

When comparing labeled molecules of differing molecular weights, the rates of macromolecular tumbling must be corrected for the smaller one. This is done using the Stokes-Einstein equation,

$$\tau_R = \frac{4\pi \eta a^3}{3kT} \quad (1.6)$$

where $\tau_R$ is the rotational correlation time, $\eta$ is viscosity, $a$ is molecular radius, $k$ is the Boltzmann constant, and $T$ is the absolute temperature. This can be rearranged to

$$\tau_R = \frac{MV\eta}{RT} \quad (1.7)$$

where $M$ is the molecular weight, $V$ is the partial specific volume, and $R$ is the universal gas constant. If $\tau_R$ for two proteins are set equal, assuming the proteins are approximately of
Figure 1.11: Hyperfine splitting of the electron spin energy levels by the nuclear spin of the nitrogen nucleus.

The electron spin is split into three lines because of interactions with the nitrogen nucleus (I=1). This diagram is taken from Knowles et al. (1976).
Figure 1.11.
Figure 1.12: A. The axes of orientation for the ππ orbitals surrounding the nitroxide. B. ESR spectra of a nitroxide spin label taken the axes described above.

The fact that spectra taken along different axis are different is the reason that ESR is so sensitive to motion. As motion of the label slows, individual orientational components have a larger impact on the overall spectrum. Taken from Giffith et al. (1976).
Figure 1.12.
Figure 1.13: The effect of increasing viscosity on the ESR spectrum of a nitroxide spin label. This figure is taken from Berliner and Shen (1977a).
Figure 1.14: Measurement of $2T_1$ (difference in field position of the arrows indicating the hyperfine extrema) from an immobilized nitroxide ESR spectrum.
similar shape, equation 1.7 becomes

\[ M_1 V_1 \eta_1 / RT = M_2 V_2 \eta_2 / RT \]  

(1.8)

where the subscripts refer to molecules 1 and 2. This equation collapses to

\[ \eta_2 = M_2 \eta_1 / M_1 \]  

(1.9)

Thus, the viscosity of the smaller protein can be adjusted to be equal to the ratio of the molecular weights to correct the rotational rotation of the smaller or equal to the larger protein.

1.3.2 Difference Absorption.

The absorption properties of chromophores, both intrinsic and extrinsic, can be a useful probe in determining the environment surrounding the chromophore. Although not as sensitive as fluorescence (which will be discussed in section 1.3.3), absorption spectroscopy is simpler and thus amenable to more direct conclusions. The instrumentation requirements are also less stringent. In this work, absorption spectroscopy is used in both functional (e.g. to monitor the production of a chromophore) and binding assays (e.g. difference absorption of proflavin).
1.3.2.1 Basic considerations.

Absorption spectroscopy encompasses a wide variety of transitions and energy levels, but only those associated with ultraviolet or visible light will be considered in this discussion. These energies correspond with transitions from the ground electronic state ($S_0$) to the first excited state ($S_1$). The absorption of a photon of ultraviolet or visible light (UV-vis) promotes the excitation of an electron in $S_0$ to $S_1$. The relaxation back to $S_0$ usually occurs non-radiatively through vibrational heat transfer, but can also occur through fluorescence or phosphorescence as discussed in section 1.3.3. This process is diagramed in Figure 1.15. Each electronic state contains many possible vibrational states ($v$). Because spectroscopy is done at temperatures higher than absolute zero, the ground state molecules exist in a distribution of vibrational states. UV-vis spectroscopy involves transitions from many different vibrational states. This, coupled with broadening due to solvent collisions tend to make the resulting transitions generally quite broad.

Because the absorption phenomenon occurs on a very short time-scale ($> 10^{15}$ s$^{-1}$), UV-vis absorption spectroscopy is sensitive to changes in the $S_0$ electronic state only (Ingle and Crouch, 1988). Changes in the $S_1$ state occur on a longer time scale and are utilized in fluorescence (discussed in section 1.3.3). For this reason UV-vis absorption is generally quite insensitive to differences in environment and is usually employed in quantitative studies utilizing Beer's law, in which $A$ is the

$$A = ecl$$

(1.6)

absorbance, $e$ is the extinction coefficient for the chromophore at that wavelength, $c$ is the
Figure 1.15: Energy level diagram of the absorption phenomenon.

Absorption of a photon of light induces the electron to be promoted from the ground electronic state $E_0$ to the first and second electronic states $E_1$ and $E_2$ (termed $S_0$, $S_1$, and $S_2$, respectively, in the text). Taken from Skoog (1985).
Absorption

Nonradiative relaxation

Figure 1.15.
concentration of chromophore, and \( l \) is the path length of the cell. In these studies, the concentration of a product of the reaction of interest is monitored by the absorbance of the solution. This method is appropriate when the substrate absorption at this wavelength is negligible.

1.3.2.2 Proflavin studies.

Chromophores which undergo large changes in their absorption spectra upon binding to proteins can be very useful probes in conformational studies. Such is the case with proflavin, which undergoes a large (~20 nm) red shift in absorption maximum upon binding to \( \alpha \)-thrombin. Because of this, proflavin difference spectra (subtraction of free proflavin spectra from bound) have provided a useful method of monitoring proflavin binding to \( \alpha \)-thrombin (Koehler and Magnusson, 1974). The peak to peak height of the difference spectra (\( \Delta A_{468\text{nm}} - \Delta A_{440\text{nm}} \)) provides a means of calculating the bound proflavin concentration (\( \epsilon_{\Delta A_{468-440\text{nm}}} = 2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \), Shen, 1977). Since several apolar ligands such as indole and ATP have been found to compete with proflavin for \( \alpha \)-thrombin, this technique can also be used to calculate dissociation constants these ligands (Shen, 1977).

Because of the exact locus of proflavin binding was not known until recently (Nienaber and Berliner, unpublished results), proflavin binding information was not amenable to specific conclusions about the \( \alpha \)-thrombin active site. However, it was known that proflavin bound within the fibrinogen groove near the active site (Sonder and Fenton, 1984). Recently the crystal structure of proflavin bound to \( \alpha \)-thrombin was solved (Nienaber and Berliner, 1996) and is shown in Figure 1.16. From this, proflavin was found to reside
Figure 1.16: The 3.0 Å resolution crystal structure of proflavin bound to human α-thrombin.

This structure was obtained by soaking of α-thrombin crystals in proflavin solution. Proflavin was observed to bind α-thrombin in the S1 pocket with hydrogen bonds between proflavin NH$_2$ and a carboxyl oxygen of Asp$^{189}$ (3.1 Å) and a hydrogen of the central nitrogen and the backbone carbonyl of Gly$^{219}$ (2.6 Å). The structure was obtained from Dr. Vicki L. Nienaber (Nienaber and Berliner, 1996).
Figure 1.16.

proflavin
between hydrogen of the central nitrogen of proflavin and the backbone carbonyl of Gly219 in the S1 specificity pocket of α-thrombin. Two important hydrogen bonds were also observed between one proflavin NH2 and a carboxylate oxygen of Asp189 and another.

1.3.3 Fluorescence.

Fluorescence spectroscopy is a powerful tool for the study of proteins and their interactions with ligands. Fluorescence is extremely sensitive to the environment around the fluorophore, whether that fluorophore is intrinsic to the protein (tryptophan, tyrosine, or phenylalanine) or an extrinsic probe (e.g. bis-ANS, DAPA, etc.). Intrinsic fluorescence tends to give more global information about the protein because it is a sum of the fluorescence emitted by each of the fluorescent residues (mainly tryptophan), while extrinsic probes are specific for the environment around the probe. In this section, a brief introduction to fluorescence and fluorescent techniques will be presented.

1.3.3.1 Basic considerations in fluorescence.

Fluorescence is defined as the stimulated emission of a photon of light between electron energy levels of the same spin multiplicity (Lakowicz, 1983). A pictorial representation is given in Figure 1.17 in the form of a Jablonski diagram. This diagram depicts three electronic states, the ground state \((S_0)\), the first excited state \((S_1)\), and the \(n\)th excited state \((S_n)\). Each electronic state contains many vibrational states, depicted here as \(v\). Upon absorption of a photon of light \((hv_{\lambda})\) the electron is excited to a vibrational state in \(S_1\). The electron then non-radiatively relaxes to the ground vibrational state of \(S_1\) on a time scale of \(10^{12} \text{ s}^{-1}\). The resulting accumulation of the \(S_1\) ground vibrational state results in a
stimulated emission of a photon of energy ($h\nu_F$) and the relaxation of the electron to $S_0$. This process, one of several possible pathways for the electron relaxation, is termed fluorescence.

If the energy of the incident photon is high enough, the electron may be excited directly up to $S_2$ in this case the electron may non-radiatively relax not only to the ground vibrational state of $S_2$, but may relax to a vibrational level of $S_1$ non-radiatively as well. This is termed internal conversion and is also depicted in the Jablonski diagram. Another possible relaxation pathway for the electron is to relax from $S_1$ to the lower energy triplet state ($T_1$). This is of a much slower time scale ($10^4$ - $10^5$ s$^{-1}$) and is termed intersystem crossing. The resulting emission upon relaxation to $S_0$ is phosphorescence ($h\nu_P$). The $S_1$ state may also depopulate by non-radiative vibrational relaxation and external conversion in the presence of quenchers.

1.3.3.2 Effects of Quenching.

As mentioned in the previous section, the excited state can decay through a variety of processes, with only some of them being radiative. This rate of decay can be described as follows:

$$k = k_f + k_i + k_{et} + k_{st} + k_q[Q]$$ (1.10)

where the rate constants $k$, $k_f$, $k_i$, $k_{et}$, $k_{st}$, and $k_q$ represent the decay of $S_1$, fluorescent radiative decay, internal conversion to thermal energy, energy transfer, intersystem crossing, and collisional quenching and $[Q]$ is the concentration of quencher. Since only one of these rates ($k_f$) results in fluorescence emission, the fraction of decay which results in fluorescence can
Figure 1.17: Jablonski diagram of the processes involved in fluorescence. This diagram is taken from Skoog (1985).
Singlet excited states

Triplet excited state

Internal conversion

Vibrational relaxation

Intersystem crossing

Absorption

Fluorescence

Phosphorescence

Vibrational relaxation

Ground state

Figure 1.17.
be described as

\[ q = \frac{k_f}{k} \]  \hspace{1cm} (1.11)

where \( q \) is the quantum yield. In the absence of quencher ([Q] = 0), the quantum yield \( (q_0) \) becomes

\[ q_0 = \frac{k_f}{k_f + k_i + k_{et} + k_{st}} \]  \hspace{1cm} (1.12)

Thus, a ratio of \( q_0 / q \) is given as:

\[ \frac{q_0}{q} = \frac{k_f}{k_f + k_i + k_{et} + k_{st}} \]  \hspace{1cm} (1.13)

which simplifies to:

\[ \frac{q_0}{q} = 1 + \frac{k_{et}[Q]}{k_f + k_i + k_{et} + k_{st}} \]  \hspace{1cm} (1.14)

The constant \( K_{sv} \) can be defined as

\[ K_{sv} = \frac{k_q}{k_f + k_i + k_{et} + k_{st}} \]  \hspace{1cm} (1.15)

Because the fluorescence intensity (F) is proportional the quantum yield (q) and substituting

\( K_{sv} \) gives:
\[ \frac{F_0}{F} = \frac{q_0}{q} = 1 + K_{sv}[Q] \]  \hspace{1cm} (1.16)

This is the Stern-Volmer equation for a single emitting fluorophore. Note that the reciprocal of the fluorescence intensity is proportional to the concentration of quencher. Thus, Stern-Volmer plots \((F_0/F \text{ vs. } [Q])\) are expected to be linear for this case. \(K_{sv}\) is very much dependent on the lifetime of the excited state and the efficiency of quenching. The longer this lifetime is the more likely the excited state is to be quenched (Eftink, 1991). In the case in which two populations of fluorophores are quenched the following equation can be used if both populations only experience collisional quenching.

\[ \frac{F_0}{F} = \frac{P}{(1+K_{sv1}[Q])} + \frac{1-P}{(1+K_{sv2}[Q])} \]  \hspace{1cm} (1.17)

where \(P\) is the fraction of quenching due to the first population, \(K_{sv1}\) and \(K_{sv2}\) are the Stern-Volmer quenching constants of populations first and second populations, respectively.

The three quenchers of relevance to this work are water, acrylamide, and iodide. Water effects its quenching through the lone pair electrons present on the oxygen atom. Because of this, fluorophores buried within proteins (unless they are exposed to another quencher such as a disulfide bridge) generally have higher quantum yields than more exposed fluorophores. Acrylamide is used in quenching experiments in this work. The fluorescence intensity of a fluorophore is monitored upon the addition of acrylamide from a concentrated stock. This intensity data, after corrected for dilution and the inner filter effect, can be plotted and fit to either equation 1.16 or 1.17. The data is then corrected for absorption by
acrylamide of the exciting light by equation 1.18, where \( F_{\text{corrected}} \) is the
\[
F_{\text{corrected}} = F_{\text{observed}} \times \text{antilog}_{10}(\text{OD} / 2)
\] (1.18)
corrected fluorescence intensity, \( F_{\text{observed}} \) is the observed fluorescence intensity previously corrected for dilution, and \( \text{OD} \) is the optical density of the solution at the exciting wavelength. Iodide also is utilized in this work as a quencher of both intrinsic and extrinsic fluorescence. While analogous to acrylamide quenching, iodide preferable quenches fluorophores which exist in the vicinity of a positive charge. This makes fluorophores near the anion exosites of \( \alpha \)-thrombin especially susceptible to quenching. Since iodide is charged, care must be taken to keep the ionic strength constant as to not change the conformation of the protein. As with acrylamide, if the protein specifically binds the quencher, then conformational changes of the protein may interfere with the quenching experiment.

1.3.3.3 Effects of polarity.

In the \( S_1 \) electronic state, the dipole moment is generally increased in magnitude. Thus, the \( S_1 \) energy level is altered by polarity of the fluorophore environment. A polar environment will stabilize the \( S_1 \) state, lowering its energy. The resulting fluorescence transition will therefore be of lower energy and the emitted photon will be of higher wavelength. This is termed a red shift and is indicative of a shift in the fluorophore environment to a more polar one (e.g. movement of a buried tryptophan to the surface of a
protein). On the other hand, a decrease in polarity will destabilize $S_1$, resulting in a blue shifted emission spectrum (e.g. the folding of a protein). This blue shift is generally accompanied by an increase in intensity due to reduced solvent quenching (Lakowicz, 1983).
CHAPTER II

THE ACTIVE SITE OF MEIZOTHROMBIN(DESF1)

2.1 INTRODUCTION

2.1.1 Goals of the study.

The role of meizothrombin (mzIIa) and meizothrombin(desF1) (mzIIa-F1) in the clotting cascade is still under study. The fact that both of these intermediates possess activity toward protein C and have limited clotting activity suggests an anticoagulant role for these enzymes. However, little is known about their structure and how this relates to their function. In fact, only recently has it been shown that they are physiological intermediates in the conversion of prothrombin to α-thrombin (Bovill et al., 1995)

MzIIa-F1 poses an interesting structural problem. It possesses less than 10 percent of the clotting activity of α-thrombin and full anticoagulant activity. Yet α-thrombin bound non-covalently to prothrombin fragment 2 (F2) demonstrates all of the properties of α-thrombin (Doyle and Mann, 1990). How does the linkage, which exists on the opposite face of α-thrombin effect such a dramatic change in function? It has also been found that the F2 moiety of mzIIa-F1 is present in roughly the same locus as the non-covalent form (Martin et al., 1993; Arni et al., 1993). What the structural differences are between α-thrombin and mzIIa-F1 which are responsible for this is very much still a mystery at this time.
It is clear from the small substrate activities of mzIIa-F1 that the active site is fully functional, but what is the nature of the residues adjacent to the active site and other exosites? Antibody binding studies have shown that anion exosite I of mzIIa-F1, mzIIa and even prothrombin is capable of binding substrates (Ni et al., 1993; Wu et al., 1994), while crystallographic studies have shown that this exosite I is not sterically occluded in prothrombin (van de Locht et al., 1996). This site is also necessary for thrombomodulin binding (Noé et al., 1988; Wu et al., 1991; Ye et al., 1992), of which both mzIIa and mzIIa-F1 are capable (Wu et al., 1992). Therefore, it is unlikely that this structural difference is present in this region of the enzyme.

A second site which is much less studied in α-thrombin, but very critical in fibrinogen binding is the group of apolar residues in and around the S3 subsites of α-thrombin (Martin et al., 1992). These reside on the opposite side of the active site and are relatively proximal to the F2 binding domain (Bode et al., 1992). Virtually nothing is known about the nature of this region of mzIIa and mzIIa-F1. Even in α-thrombin, this site is not nearly as well studied as the anion exosites. The goal of this study is to decipher the differences in the solution structure of mzIIa-F1 (and mzIIa to a lesser extent) using biophysical techniques and determine how these differences manifest themselves in the physiological activities of the enzymes.

2.1.2 Rationale of the approach and summary of the results.

Both ESR and fluorescence present excellent, complementary techniques for examining the solution structure differences between mzIIa-F1 and α-thrombin (Berliner,
Figure 2.1 Spin labels used in this study. Chiral carbons are indicated by an asterisk (*).
A series of sulfonylfluoride labels, shown in Figure 2.1, developed in the laboratory of Dr. L. J. Berliner have been used for characterizing serine proteases, specifically chymotrypsin and α-thrombin (Wong et al., 1974; Berliner and Wong, 1974; Berliner and Shen, 1977a). These labels aided many successful predictions about the structure of α-thrombin (of which no crystal structure existed until 1989) such as the prediction of an apolar site (Berliner and Shen, 1977b) and an anion binding region (Berliner et al., 1985). Therefore, similar types of predictions can be made of mzIIa and mzIIa-F1, of which no crystal structures are currently published. Fluorescence is also extremely sensitive to differences in structure and can provide both global and local information about the fluorophore. Both intrinsic and extrinsic studies of mzIIa-F1 and α-thrombin give information about the exposure of tryptophans and fluorescent active site probes (Krishnaswamy et al., 1986). Fluorescence also can provide information which is independent of molecular weight, unlike ESR, which is affected by macromolecular tumbling rates.

The motions of the ESR spin labels attached to mzIIa-F1 revealed that para labels were found to be significantly different in mobilities than those of α-thrombin. These labels most likely probe the S2 and S3 positions of mzIIa-F1, based on the recently obtained crystal structure of p-V α-thrombin (Nienaber et al., 1996). These labels suggest that the differences in the α-thrombin and mzIIa-F1 structures are in the apolar region in the vicinity of S2 and S3 (possible more distant as well). This may very well be the cause of the reduced fibrinogen affinity, thus responsible for the anticoagulant nature of mzIIa-F1. The meta labels, on the other hand, gave strikingly different results. These were found previously to
exhibit changes in mobility upon conversion from α-thrombin to γ-thrombin [cleavage of Lys 1496 to free the autolysis loop rendering the anion exosite I functionally inactive (Berliner et al., 1981; Berliner, 1984; Berliner et al., 1986)]. All of the meta labels, with the exception of m-VII, revealed little or no differences in mobility between the mzIIa-F1 and α-thrombin bound forms. This data indicates that the autolysis loop and the general region of the active site are quite similar between the two enzymes.

Benzamidine is a competitive inhibitor of α-thrombin, which has been found to bind the S1 specificity pocket of α-thrombin (Stüzebecher et al., 1984; Bode et al., 1990). The effect benzamidine on spin labeled α-thrombin and mzIIa-F1 was to immobilize the meta labels on both enzymes. An immobilization of about 5 G was noted for each label. A dissociation constant of $1.0 \pm 0.1 \text{ mM}$ was calculated for spin labeled α-thrombin and mzIIa-F1. However, for the para labels $p$-I and $p$-V, no immobilization was noted. This provides evidence that the general active site region (around S1) of both enzymes is roughly similar in conformation. These results also agrees with crystal structure data, which places m-V in the S1 specificity pocket and p-V away in S2, presumably unaffected by occupation of the S1 pocket.

The binding of indole was found to affect label mobility as well. The presence of 20 mM indole effects a change in mobility of labels m-VII, $p$-I, and $p$-V on α-thrombin, as well as m-VII and $p$-I on mzIIa-F1. It is of interest to note that changes invoked by indole are somewhat different for mzIIa-F1 vs. α-thrombin. For example, the presence of 20 mM indole results in a modest 3 G increase in $2T_1$ for m-VII labeled α-thrombin, but the same ligand causes a dramatic 11 G increase in m-VII labeled mzIIa-F1. Esterase activity studies
also demonstrate that mzIIa-F1 is not activated by indole as α-thrombin is. All of the above data suggest that the indole binding site of mzIIa-F1 is present, but does not evoke the same conformational changes as it does on α-thrombin.

Fluorescence studies utilizing both intrinsic and extrinsic fluorophores have revealed similar findings to those found by ESR spin labels. Intrinsic α-thrombin as well as dansyl α-thrombin, which resides directly in the active site, show little difference in fluorescent properties from the corresponding mzIIa-F1. However, both DAPA and dansyl-EGR-CH₂ labeled mzIIa-F1 show blue shifted emission spectra and differences in iodide quenching when compared to the corresponding α-thrombin spectra. The fluorescence data suggest that the active site groove in the S2-S6 region of mzIIa-F1 is more occluded than that of α-thrombin.

2.2 MATERIALS AND METHODS

2.2.1 Materials.

Crude ECV, ecarin, indole (lot 36C0204), DEAE-cellulose (lot 25C-0174), benzamidine hydrochloride (lot 022567) Amberlite CG-50 (lot 90H0250), and Tris-base were purchased from Sigma Chemical Co. Proflavin hydrochloride (lot 11335) was obtained from Allied Chemical. Proflavin was recrystallized in ethanol according to Conery (1981) prior to use. PPACK (lot 504292) and dansyl-EGR-CH₂Cl (lot 056012) were from CalBiochem, while ammonium persulfate (lot 119913C), TEMED (lot 466358), Coomassie brilliant blue (lot M4203), SDS (lot 121016D), and P-6 Biospin columns were purchased from Bio-Rad. DOPC was purchased from Avanti Polar Lipids. S-2288 and S-2366 were
purchased from Kabi Diagnostics, while chromothrombin was a generous gift from Diagnostica Stago. Sulfonylfluoride spin labels were synthesized previously as per Wong et al. (1974). DAPA was synthesized according to Nesheim et al. (1979). All other chemicals were obtained as reagent grade or better and used without further purification.

Bovine \(\alpha\)-thrombin (lot J10603) was received in lyophilized form as a generous gift from Armour Pharmacueticals. TMD1(CS+) and TMD1(CS-) were prepared as recombinant proteins by Bruce Gerlitz and Dr. Brian Grinnell at Eli Lilly and Company in Indianapolis, IN.

### 2.2.2 Proteins

#### 2.2.2.1 Purification of bovine prothrombin.

Bovine plasma was obtained from Dr. Dan Walz, Wayne State University (Detroit, MI). Plasma was thawed and 15 mL of 1 M barium chloride was added for every 1 L of plasma. After stirring overnight at 4 °C, the barium citrate precipitate was collected by centrifugation for 30 min at 3600g. The pellet was then washed with citrate buffered saline (26 mM sodium citrate, 0.9% NaCl) and stored at -70°C until further purification. The pellet was dissolved into 120 mL/ L plasma of 0.2 M EDTA, pH 8.0 and an equal volume of citrate saline. The solution was then brought to 10% saturated ammonium sulfate and stirred overnight (at 4°C). The precipitate was then removed by centrifugation (30 min at 3600 g) and the supernatant was made to 40% ammonium sulfate. This was stirred for 20 min and centrifuged for 30 min at 3600 g. Finally the supernatant was made to 60% ammonium sulfate and, after centrifugation (30 min at 3600 g), the resultant pellet was redissolved in a
minimum volume of 25 mM citrate, pH 6.0 and dialyzed overnight vs. the same buffer. The solution was then centrifuged again for 20 min at 3600 g and applied to a DEAE-cellulose column equilibrated in the same buffer. The column was washed until the resulting absorbance at 280 nm was < 0.02 and a 1500 mL gradient of 0 - 0.5 M NaCl was applied. Prothrombin eluted at approximately 0.3 M NaCl. The resulting peak was pooled and dialyzed vs. 0.75 M NaCl (unbuffered).

2.2.2.2 Generation of Bovine Meizothrombin(desF1).

Eight mLs of bovine prothrombin in 20 mM Tris, 0.15 M NaCl, 5.0 mM CaCl$_2$, pH 7.4 were activated to meizothrombin (desF1) by the addition of 200 μL of 10 U/ml ecarin, a protease from the venom of *Echis carinatus* which catalyzes the cleavage on the C terminal side of Arg$^{330}$ (Franza *et al.*, 1975; Morita *et al.*, 1976; Breit *et al.*, 1982). Activation was monitored by chromogenic activity. When activation was complete (2-3 hours), the crude meizothrombin(desF1) was passed through 2 mL of DEAE-cellulose to remove an unreacted prothrombin and fragment 1. Some of the resulting meizothrombin(desF1) was later passed through Amberlite CG-50 (in 0.15 M NaCl) to remove any thrombin present.

2.2.3 Electrophoresis.

Electrophoresis was generally performed on a Bio-Rad mini-Protean system. All gels used in this study were 12 % acrylamide and some were silver stained according to Merril *et al.* (1983). Gels were typically run at a constant voltage of 180 V with a current in the range of 30 mA for 45 min to an hour. Coomassie staining consisted of soaking the gel in
40% methanol, 10% acetic acid containing 0.1% Coomassie brilliant blue for 30 - 60 min. Gels were then destained in the same solvent minus the dye for 1 hour and then destained in 10% methanol, 10% acetic acid until desired staining was obtained. Gels were often dried with a Bio-Rad gel drier for 45 min at 60°C under vacuum.

2.2.4 ESR Spectroscopy.

2.2.4.1 Spin labeling.

α-Thrombin or mzIIa-F1, stored in 0.75 M NaCl, was spin labeled with m-IV, m-V, or m-IV p-I, p-IV, p-V by dialyzing vs. 20 mM Tris, 0.15 M NaCl, pH 6.5 for 2 h. The pH of the solution was then adjusted to 7.4 with 1.0 M Tris-base. Spin label (in acetonitrile) at a concentration of 10 fold excess to the protein, was then added. The amidolytic activity (or esterase) of the solution was then monitored until greater than 95% inhibition was noted (usually 1-2 hours). The reaction was then stopped with either 20 fold tosyl fluoride (in acetonitrile) or 2 fold PPACK, a chloromethyl ketone inhibitor which covalently reacts with His57 (Kettner and Shaw, 1979; Williams and Mann, 1993). Free spin label was then removed by exhaustive dialysis vs. 50 mM Tris, 0.75 M NaCl, pH 6.5.

Alternatively, Bio-Rad P-6 polyacrylamide columns were employed to remove free label. In this case, the columns were washed with 3 aliquots of 300 µL of 50 mM Tris, 0.75 M NaCl, pH 6.5 by centrifuging at medium speed with a clinical centrifuge. The sample (no more than 100 µL per column) was then applied and spun at medium speed for 2 min. Columns were generally reused once or twice.
2.2.4.2 ESR Spectroscopy.

ESR spectroscopy was performed on either a Varian E-9 or E-12 at room temperature. Typical instrument parameters ranged from a gain, $5 \times 10^3$ to $6.3 \times 10^4$; modulation 0.5 G to 2.5 G; scan time 8 min to 2 hours; time constant 0.3 s to 1.0 s; power, 20 mW to 30 mW; scan range, 100 G; and center field, 3400 - 3300 G. Protein concentrations were generally in the range of 10 - 30 μM. Concentrations of spin label were determined using the spin count method discussed in Chapter 1. Unless specified in the figure, samples were measured in a 150 μL flat cell.

2.2.5 Fluorescence Spectroscopy.

2.2.5.1 Fluorescence labeling.

Dansyl labeled α-thrombin was prepared by incubating a 5-10 μM solution of α-thrombin or mzIIa-F1 in 20 mM Tris, 0.15 M NaCl, pH 7.4 with 300 μM dansyl fluoride (see figure 2.16) in isopropanol (isopropanol does not exceed 10%) for 5 hours at room temperature. The activity of the α-thrombin was monitored by an amidolytic assay. α-Thrombin was then placed in the cold room to stir for 3 days at 4 °C. Typical labeling efficiencies ranged from 30-60%. Electrophoresis was used to ensure that no degradation of the α-thrombin had occurred. Labeling with dansyl-EGR-CH₂Cl was accomplished by incubation with 5-fold excess of label followed by exhaustive dialysis vs. 0.75 M NaCl (unbuffered).
2.2.5.2 Fluorescence Spectroscopy.

Fluorescence spectroscopy was performed on a Perkin Elmer LS-50B spectrofluorometer. Slit widths were typically 6.0 to 10.0 nm and the temperature controlled by a VWR Scientific Model 1141 temperature controller to ±0.1 °C. An emission filter of 470 nm was also employed for all dansyl measurements to remove intrinsic fluorescence signal unless otherwise noted.

2.2.5.3 Fluorescence quenching studies

Acrylamide quenching studies were performed by the addition of aliquots of a 5.0 mM acrylamide solution to a cuvette containing 3.00 mL of the DAPA-α-thrombin or dansyl α-thrombin. The absorbance of the solution at the exciting wavelength after each addition was also recorded to correct for filtering of the excitation light by the acrylamide using equation 1.18.

2.2.5.4 Thrombomodulin studies

For thrombomodulin experiments, approximately 500 nM labeled α-thrombin was titrated with TMD1(CS+) to a five fold excess. Cuvettes were soaked overnight in a sonicated solution of 400-500 μM DOPC in 50 mM HEPES, 70 mM NaCl, pH 7.5 before use to prevent α-thrombin adsorption onto the cell walls. The excitation and emission wavelengths were 280 nm and 535 nm, respectively. An excitation wavelength gave identical results to that of 280 nm. However, a wavelength of 280 nm was used because of increased signal intensity.
2.2.6 Proflavin binding.

The binding of proflavin to α-thrombin and meizothrombin(desF1) was monitored by a change in absorption maximum between the bound proflavin (468 nm) and the free proflavin (444 nm). Proflavin difference spectra were obtained by subtraction of the free proflavin spectra from bound. Concentration of bound complex can also be calculated form the peak to peak height of this difference spectrum \((\Delta A_{468} - \Delta A_{444}; \epsilon = 2.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1})\). Ligands can then be added to compete off the proflavin. The buffer used was 20 mM HEPES, 0.15 M NaCl, pH 7.0. Spectra were obtained on a Uvikon 960 spectrophotometer (Kontron Instruments).

2.2.7 Activity Assays.

2.2.7.1 Amidolytic Assays

Substrates S-2288 (H-D-isoleucyl-L-prolyl-L-argininyl-p-nitroanilide), S-2366 (L-glutamyl-L-prolyl-L-argininyl-p-nitroanilide), and chromothrombin (tosyl-L-glycyl-L-prolyl-L-arginine-p-nitroanilide) were used to measure the amidolytic activities of α-thrombin and mzIIa-F1 (Lottenburg et al., 1982; Lottenburg et al., 1983). A 1.5 mM stock solution was prepared for each substrate. To a cuvette, 900 μL of assay buffer (50 mM Tris, 100 mM NaCl, pH 8.3) was added along with 100 μL of substrate. The assay was also performed at times using 990 μL and 10 μL of buffer and substrate, respectively. Ideal α-thrombin concentration was approximately 5 pM. The change in absorbance at 405 nm was measured upon addition of α-thrombin.
Alternatively, some assays were performed on a Molecular Devices plate reader. For these assays, a disposable 96-well plate was used with a total volume of 200 μL per well. 100 μL of buffer and 50 μL of ~20 pM α-thrombin were added to each well. The assay was initiated by the addition of 50 μL of 30 μM substrate (concentration may be varied) to each well with a multipipette.

2.2.7.2 TAME assay in the presence of Indole.

Since indole interferes with the spectrophotometric measurement of released tosylate, this assay must be performed by the measurement of NaOH necessary to maintain a constant pH. Six aliquots of (100 mLs each) 0.4 mM Tris, pH 8.1 are prepared containing 0, 4, 8, 12, 16, or 20 mM indole. Ten mLs of this buffer are then added to a pH-stat cup and equilibrated to 28°C. The buffer is then placed into Radiometer pH-stat with argon bubbling through it to prevent any pH changes due to dissolved CO₂. A final concentration of 50 nM of α-thrombin (or meizothrombin(desF1)) is then added and the amount of 0.10 M NaOH required to keep the pH constant is then recorded by the instrument. The slope of this line is then proportional to the activity. This was repeated for each of the indole concentrations for both α-thrombin and meizothrombin(desF1).

2.2.7.3 Active site assay

The NPGB (p-nitrophenyl-p-4'-guanidinobenzoate) assay was performed to determine the number of active sites available on α-thrombin. Both sample and reference cuvettes contain 1000 μL of 50 mM barbital, 0.15 M NaCl, pH 8.3 and 5 μL of 10 mM NPGB in 1:4
DMF:acetonitrile. α-Thrombin concentrations range from 1 to 7 μM. Once α-thrombin was added to the sample cuvette, a burst phase and acylation phase are observed while monitoring the absorbance at 410 nm. The acylation was then extrapolated back to time zero. This absorbance was used to calculate the concentration of active sites.

2.3 RESULTS

2.3.1 ESR Studies of the conformation of mzIIa-F1.

In order to obtain information about the conformation of the mzIIa-F1 active site, bovine α-thrombin and mzIIa-F1 were spin labeled with the following paramagnetic labels: m-IV, m-V, m-VII, p-I, p-IV, and p-V. The resulting 2T₁ of the X-band ESR spectra of mzIIa-F1 and α-thrombin are given in Table 2.1. Because the molecular weight of α-thrombin is approximately half that of mzIIa-F1 (37,000 vs. 52,000 daltons) the ESR spectra of α-thrombin must be corrected for the difference in rotational rates, accomplished by increasing the viscosity with sucrose as described in section 1.3.1.3. With the exception of m-VII, the resulting 2T₁'s for the labels in which the sulfonylfluoride moiety is in the meta position (meta labels; designated with an m; Berliner and Wong, 1974) were generally quite similar for α-thrombin and mzIIa-F1 (see Table 2.1). Therefore, the environments surrounding the label in both α-thrombin and mzIIa-F1 are similar and thus the conformation of the region of the α-thrombin active site which these labels probe are comparable as well.

On the other hand, the para labels present a different picture with p-IV and p-V showing the most dramatic results with changes in 2T₁ of 8.4 and 9.1 G, respectively. The p-IV results are most persuasive because this label became more mobile in mzIIa-F1, leaving
Figure 2.2 X-band ESR spectra for m-IV labeled bovine α-thrombin in the presence of a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, or d) 12 % (w/v) sucrose.

α-Thrombin concentrations were from 25 - 50 μM. The buffer was 50 mM Tris, 0.75 M NaCl, pH 6.5, 25 ±2°C. Arrows indicate the hyperfine extrema.
Figure 2.2.
Figure 2.3 X-band ESR spectra of m-IV labeled mzlα-F1 in a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, or d) 3 mM CaCl₂. Conditions are identical to those in Figure 2.2.
Figure 2.3.
**Figure 2.4** X-band ESR spectra of $m$-V labeled bovine a) $\alpha$-thrombin, b) $\alpha'$-thrombin in 12% sucrose, and c) mzIIa-F1. Conditions are identical to those in Figure 2.2.
Figure 2.4.
Figure 2.5  X-band ESR spectra of m-VII labeled bovine α-thrombin in a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, or d) 12 % (w/v) sucrose. Conditions were identical to those in Figure 2.2.
Figure 2.5.
Figure 2.6 X-band ESR spectra of $m$-VII labeled bovine mZIIa-F1 in a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, or d) 5 mM CaCl$_2$. Conditions were identical to those in Figure 2.2.
Figure 2.6.
Figure 2.7 X-band ESR spectra of p-I labeled bovine α-thrombin in a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, or d) 12% (w/v) sucrose. Conditions were identical to those in Figure 2.2.
Figure 2.7.

\[
\begin{align*}
\text{a)} & \quad \text{(p-NH-5=CO)} \\
\text{b)} & \\
\text{c)} & \\
\text{d)} & 
\end{align*}
\]
Figure 2.8 X-band ESR spectra of p-I labeled bovine mzIIa-F1 in a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, or d) 5 mM CaCl₂. Conditions were identical to those in Figure 2.2.
Figure 2.8.
**Figure 2.9** X-band ESR spectra of p-IV labeled bovine a) α-thrombin, b) α-thrombin in 12% (w/v) sucrose, and c) mzIIa-F1. Conditions were identical to those in Figure 2.2.
Figure 2.9.
Figure 2.10 X-band ESR spectra of $p$-V labeled bovine $\alpha$-thrombin in a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, or d) 12 % (w/v) sucrose. Conditions were identical to those in Figure 2.2.
Figure 2.10.
Figure 2.11 X-band ESR spectra of p-V labeled bovine mZIIa-F1 in a) buffer, b) 2.5 mM benzamidine, c) 20 mM indole, d) 5 mM CaCl₂. Conditions were identical to those in Figure 2.2.
Figure 2.11.
TABLE 2.1

Observed $2T_1$ values (± 0.5 G) for spin labeled α-thrombin (column 1), meizothrombin(desF1) (column 2), and α-thrombin corrected for molecular weight differences with 12% sucrose (column 3). Buffer is 50 mM Tris, 0.75 M NaCl, pH 6.5. Note, little variation between α-thrombin and mzIIa-F1 is present for the meta labels, while para labels show a significant difference.

<table>
<thead>
<tr>
<th>Label</th>
<th>α-thrombin</th>
<th>mzIIa-F1</th>
<th>α-thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(12% sucrose)</td>
</tr>
<tr>
<td>$m$-IV</td>
<td>45.7 G</td>
<td>46.9 G</td>
<td>48.8 G</td>
</tr>
<tr>
<td>$m$-V</td>
<td>61.5</td>
<td>62.3</td>
<td>61.4</td>
</tr>
<tr>
<td>$m$-VII</td>
<td>54.1</td>
<td>50.1</td>
<td>55.0</td>
</tr>
<tr>
<td>$p$-I</td>
<td>50.0</td>
<td>52.5</td>
<td>52.0</td>
</tr>
<tr>
<td>$p$-IV</td>
<td>57.3</td>
<td>49.0</td>
<td>57.4</td>
</tr>
<tr>
<td>$p$-V</td>
<td>51.9</td>
<td>62.1</td>
<td>53.2</td>
</tr>
</tbody>
</table>
Figure 2.12 Plots of $2T_1$ vs. benzamidine concentration for A. $m$-IV and B. $p$-V labeled $\alpha$-thrombin and mzIIa-F1. Conditions were identical to those in Figure 2.2.
Figure 2.12.
Figure 2.13 Superimposed crystal structures of $p$-$V$ and $m$-$V$ labeled human $\alpha$-thrombin obtained by soaking of $\alpha$-thrombin crystals in spin label.

These crystal structures were obtained by Dr. V. L. Nienaber at DuPont-Merck, Wilmington, DE (Nienaber and Berliner, 1996). The spin labels $m$-$V$ and $p$-$V$ were labeled as a racemic mixture of two isomers. The R isomer (dark gray) of $m$-$V$ was found to probe in the direction of the autolysis loop (Trp$^{148}$) and the S isomer (light gray) resides directly in the S1 specificity pocket. Both R and S isomers of $p$-$V$ lay in the fibrinogen binding groove in the S2/S3 subsites. The stoichiometry of isomer labeling is not known.
Figure 2.13.
Figure 2.14 A bar graph summary of the effect of 20 mM indole on the $T_1$ values of spin labeled bovine $\alpha$-thrombin and mzIIa-F1. The ordinate is the change in $T_1$, upon addition of 20 mM indole. Conditions were identical to those in Figure 2.2.
Figure 2.15 The effect of indole on TAME (tosyl-L-argininyl-methyl ester) esterase activity of bovine α-thrombin and mzIIa-F1. Activity was measured by the volume per unit time of 0.01 M NaOH required to neutralize the acid produced by the cleavage of TAME substrate. The constant pH was maintained by use of a pH-stat. Buffer was 0.4 mM Tris, pH 8.1, 25 ± 0.5 °C
little doubt that this was not due to molecular weight differences. The conclusion, analogous to that of the meta labels, is that the para labels probe a locus of the active site which differs in conformation between the two enzymes. Two of these labels, m-VII and p-l, provide results which are somewhat inconsistent with others in the same family. From the structures of these labels (see Figure 2.1), possible explanations for this effect can be provided. The label p-l is the only label of the group with a proxyl ring, which has been found to have more barriers to free rotation about the ring than the pyrrolidinyl and piperidinyl rings of the other labels (T. -L. Chang and Berliner, unpublished results). Thus, the nitroxide moiety of the p-l may probe a different location of the active site due to a different conformation of the label. This is supported by initial crystallography studies of p-l labeled α-thrombin done by B. F. P. Edwards' laboratory, where it was found that p-l exists in the cis conformation around the amide bond, rather than the expected trans conformation (Edwards, B. F. P., personal communication). The label m-VII, also is somewhat unique since it is somewhat longer than the other labels (approximately 3-4Å) and is therefore likely to occupy a different region of the active site than other meta labels. This has experimental support as well. In bovine α-thrombin, this label was found to give results similar to para labels, suggesting the binding locus of the nitroxide being closer to that of the para labels (Nienaber and Berliner, 1991).

The effects of ligand binding to spin labeled thrombins also provides a great deal of structural information about the enzyme. ESR spectra of α-thrombin and mzIIa-F1 labeled with m-IV, m-V, m-VII, p-I, p-IV, or p-V and in the presence of benzamidine, indole, or Ca^{2+} are given in Figures 2.2 - 2.11. Benzamidine is a reversible inhibitor of α-thrombin which is known to bind the S1 specificity pocket (Bode et al., 1990). Thus, it can also be used as
a probe of this region of the \( \alpha \)-thrombin active site. Indole most probably binds a region around S2, since this area is known to bind a hydrophobic area near the active site (Berliner and Shen, 1977a), although no direct crystallographic evidence exists. Indole does not, however, inhibit fibrinogen clotting, so it most likely does not bind directly in S2. As stated in Chapter 1, a significant amount of evidence exists that suggests there is an additional indole binding site on \( \alpha \)-thrombin, thus indole probes this site(s) as well. The F2 portion of prothrombin has been previously found to bind \( \text{Ca}^{2+} \). For this reason the effect of \( \text{Ca}^{2+} \) to spin labeled mzIIa-F was also investigated and presented in Figures 2.3, 2.6, 2.8, and 2.11.

The effects of benzamidine and indole binding to \( m \)-IV \( \alpha \)-thrombin and mzIIa-F1 are shown in Figures 2.2-2.3. For both thrombins an increase in \( 2T_1 \) of approximately 5 G was noted, indicating a decrease in the mobility of the label upon benzamidine binding. Titrations (Figure 2.12) reveal a dissociation constant of 1.0 ± 0.1 mM for benzamidine binding to both enzymes. This compares somewhat favorably to the inhibition constant of 1.4 mM found by Stürzebecher \textit{et al.} (1984). Clearly this result may be altered by the presence of spin label in the active site. The conclusion can be made, however, that mzIIa-F1 and \( \alpha \)-thrombin possess S1 specificity pockets of similar conformation and structure. This is also supported by previous activity studies, in which it was found that mzIIa-F1 possesses similar small substrate activity to that of \( \alpha \)-thrombin (Doyle and Mann, 1990). Figure 2.13A shows the crystal structure of \( m \)-V labeled human \( \alpha \)-thrombin determined by Nienaber and Berliner (1996). It is of interest to note that \( m \)-V and \( p \)-V exist as racemic mixtures because of the chiral carbons indicated in Figure 2.1. For this reason, two isomers (R and S) of \( m \)-V
are shown in the crystal structure. The R isomer is quite bent and does not fit in the active site groove. Thus, it extends toward the autolysis loop, while the S isomer of m-V resides in the S1 specificity pocket of α-thrombin. These crystal structure findings are consistent with the results given above as well as earlier work with α-thrombin (Berliner and Shen, 1977; Berliner et al., 1981; Nienaber and Berliner, 1991). Similar results were obtained for m-VII labeled enzymes. In both cases, an approximate increase in $T_1$ of 5 G was noted, concurrent with a $K_D$ of 1 mM (data not shown).

The results were somewhat different for $\beta$-I and $\beta$-V (Figures 2.7, 2.8, 2.10, and 2.11). Little if any change in $T_1$ was observed upon benzamidine binding. This is most likely indicative of minimal effects of benzamidine binding upon mobility of the label, although the absence of binding cannot be ruled out. The crystal structure of $\beta$-V bound to α-thrombin (Figure 2.13) reveals that this label binds a locus distinct from that of the S1 pocket and thus it is not surprising that label mobility is unaffected by benzamidine. It is also significant that labels on mzIIa-F1 display similar changes in the presence of benzamidine, providing empirical evidence that $\beta$-V probes analogous regions of mzIIa-F1.

The effects of indole binding to α-thrombin were somewhat different. Previous results from the Berliner laboratory have revealed that para labels are sensitive to the binding of indole to α-thrombin (Berliner and Shen, 1977b; Nienaber and Berliner, 1991). This appears to be true for mzIIa-F1 as well. Figure 2.14 summarizes the changes in $T_1$ upon addition of 20 mM indole to spin labeled α-thrombin and mzIIa-F1. The results indicate that mzIIa-F1 possesses an indole binding site (or more), since ESR spectral changes occur upon the addition of indole, although label induced indole binding cannot be ruled out. However,
the labels which undergo large changes are different. For example $p$-$V$ when bound to mzIIa-F1 undergoes no mobility change upon the addition of indole, while for $\alpha$-thrombin the label becomes more mobile. On the other hand, addition of indole to $m$-VII labeled mZIIa-F1 results in a tremendous immobilization (9 G) compared to a moderate increase (3 G) for $\alpha$-thrombin. The above results suggest that mZIIa-F1 has an apolar binding domain, which elucidates different conformational responses in mZIIa-F1. In other words, the apolar binding site of mZIIa-F1 may serve a functionally different role than that on $\alpha$-thrombin.

Another method of investigating the apolar binding domain of mZIIa-F1 is the use of activity assays. $\alpha$-Thrombin esterase activity is activated in the presence of indole in low millimolar concentrations ($K_{act \ (app)} = 11.4 \text{ mM; Conery and Berliner, 1983}$). The TAME esterase activity of $\alpha$-thrombin and mZIIa-F1 in increasing indole is shown in Figure 2.14. This clearly demonstrates that indole induces a three fold activity enhancement in $\alpha$-thrombin, none such enhancement is found with mZIIa-F1. This result lends support to the hypothesis that the mZIIa-F1 indole binding domain is functionally immature compared with that of $\alpha$-thrombin. It is interesting that despite evidence for indole binding to $\alpha$-thrombin, no such activity effect is observed. It is likely that the conformational change induced by indole is blocked in mZIIa-F1, either through a lack of "communication" between the indole and active site or indole binds in such a manner that the conformational change is no longer triggered. Either way, it is clear that indole, and presumably indole derivatives released by platelets, do not exert the same physiological effects on mZIIa-F1 as $\alpha$-thrombin.

Since F2 of prothrombin is known to bind Ca$^{2+}$ with a $K_D$ of 3.2 mM at approximately 4 sites (Bajaj et al., 1975), the effect of Ca$^{2+}$ on the mobilities of the spin
labels on mzIIa-F1 was also investigated. The ESR spectra recorded in the presence of 5 mM CaCl$_2$ are shown in Figures 2.3, 2.6, 2.8, and 2.11. No change in label mobility was observed upon the addition of Ca$^{2+}$ for any of the four label measured. Thus, it is likely that no conformational change occurs at the active site upon the binding of Ca$^{2+}$ by F2 in mzIIa-F1.

2.3.2 Fluorescence studies of mzIIa-F1.

The active site of mzIIa-F1 can also be probed with several fluorescent probes, as shown in Figure 2.16. Dansyl fluoride is attacked by the Ser$_{195}$ of $\alpha$-thrombin as indicated by the arrows in Figure 2.16, to form a covalent active site labeled fluorescent complex. Dansyl-EGR-CH$_2$Cl labels the active site as well. However, it attacks His$_{57}$ and the fluorescent moiety resides distal to the active site (around S4), thus probing a different region of the active site. Although no crystal structure of dansyl-EGR-CH$_2$Cl labeled $\alpha$-thrombin exists, the location of the dansyl moiety can be easily estimated from the structure of PPACK-$\alpha$-thrombin (Brookhaven data bank structure 2hpq.pdb). DAPA is a tightly binding reversible fluorescent inhibitor of $\alpha$-thrombin which also probes the environment of the active site. Finally, bis-ANS is a nonspecific noncovalent label which binds to hydrophobic regions of proteins (Musci et al., 1985). The fluorescence spectra of $\alpha$-thrombin and mzIIa-F1 are given in Figure 2.17. A comparison of intrinsic spectra (Figure 2.17A) shows that the emission maxima for these spectra are each at 339 nm indicating no large difference in the polarity of the tryptophan environments. Figure 2.17B consists of spectra of DAPA $\alpha$-thrombin and mzIIa-F1. In this case, two changes are apparent. One is a blue shift in the emission spectrum of DAPA-mzIIa-F1 in comparison of that of DAPA-$\alpha$-thrombin.
**Figure 2.16** Fluorescent labels used in this study. Arrows refer to bonds which are attacked by Ser^{195}.
second is a reduction in intensity. A blue shift of a decrease in the polarity in the label environment, consistent with a more occluded, buried fluorophore. The reduction in intensity must be interpreted carefully because it may be due to a change in the DAPA-mzIIa-F1 binding constant or increased exposure to a quencher (such as solvent or a disulfide bridge). In comparing the spectra of the dansyl-EGR-CH2Cl labeled thrombins, a difference of about 5 nm was noted in the emission maxima (533.7 nm vs. 529.4 nm for α-thrombin and mzIIa-F1, respectively). The blue shifted mzIIa-F1 suggests a dansyl moiety which is in a more hydrophobic environment. This result is in direct agreement with the ESR results earlier in which a difference was found in S2 and S3.

An intriguing hypothesis concerning the structural differences between α-thrombin and mzIIa-F1 is that subsites S2-S4 (at least) are less exposed in mzIIa-F1, thus unable to bind fibrinogen as well as α-thrombin. To test this, the above labeled thrombins were exposed to iodide and the fluorescence monitored (Figure 2.18). In the case of intrinsic fluorescence (Figures 2.18A and B), iodide quenching results in two-component collisional quenching (described by equation 1.17; Eftink, 1991). The results of quenching experiments are given in Table 2.2. Note that with intrinsic quenching, both quenched populations are more exposed in mzIIa-F1 than α-thrombin.

When the quenching of dansyl labeled α-thrombin and mzIIa-F1 is considered, the result is one component collisional quenching for dansyl α-thrombin (Figures 2.18C and D). In the case of mzIIa-F1, however, a very unusual result is obtained. The fluorescence is actually enhanced upon exposure to iodide. This can only be the result of a conformational change experienced by mzIIa-F1 in the presence of iodide (i.e., iodide ions binding
specifically mzIIa-F1 resulting in a conformational change which enhances dansyl fluorescence). It is also a requirement that the dansyl moiety be somewhat buried to prevent quenching from having a major effect. The $K_{sv}$ calculated from dansyl $\alpha$-thrombin supports this as it is less exposed than intrinsic quenching by more than 10-fold. Since $\alpha$-thrombin has been shown to specifically bind chloride (Vindigni and Di Cera, 1996), specific binding of iodide would not be a surprising result. It is significant that it influences the active site since chloride ions are thought to bind exosite I (Vindigni and Di Cera, 1996). It is also interesting that this effect was not seen for dansyl $\alpha$-thrombin, and becomes another interesting difference between the two enzymes.

As with intrinsic fluorescence, quenching of dansyl-EGR-$\alpha$-thrombin and mzIIa-F1 results in two-component collisional quenching when excited at either 335 nm (direct dansyl excitation) or 280 nm (energy transfer from tryptophans). Unlike dansyl $\alpha$-thrombin and mzIIa-F1 the dansyl moiety of dansyl-EGR-$\alpha$-thrombin and mzIIa-F1 is quite exposed to solvent. These results indicate that the dansyl fluorophore is not located in the fibrinogen groove, but rather is directly in solution. This is likely because conformational restrictions limit the bulky group, consistent with studies using analogous spin labels (Sankarapandi and Berliner, unpublished results). Both dansyl- and dansyl-EGR- labeled enzymes show the difference between the two thrombins. The dansyl moiety was found to be less solvent exposed in dansyl-EGR-CH$_2$-mzIIa-F1. Thus, it is likely that the S2-S4 subsites of mzIIa-F1 are more buried and less accessible in mzIIa-F1. This may even extend further to S6 given the recent discovery that mutating Glu$^{229}$ to an alanine forms an anticoagulant-thrombin with similar characteristics to mzIIa (Gibbs et al., 1995).
Figure 2.17 Fluorescence spectra of both bovine α-thrombin and mzIIa-F1 which are A. unlabeled (intrinsic), B. in the presence of 3.0 μM DAPA, and C. labeled with dansyl-EGR-CH₂Cl. Excitation wavelength for all spectra is 280 nm. Dansyl moiety is excited by energy transfer from tryptophans. Protein concentration in each case was 0.5 μM and the buffer was 20 mM Tris, 0.15 M NaCl, pH 7.0, 20 ±0.2 °C.
Figure 2.17.
Figure 2.18 Stern-Volmer quenching curves of A. and B. intrinsic C. and D. dansyl, and E. - H. dansyl-EGR-CH2Cl labeled α-thrombin and mzIIa-F1. Plots A, C, E, and G depict the fluorescence intensity decreasing with increasing iodide, while plots B, D, F, and H show the Stern-Volmer plots of each. Excitation wavelengths were 280 nm for A, B, G, and H, while 335 nm was employed for C - F. Slit widths were 6.0 nm for A and B, and 10.0 nm for C - H. Buffer was 20 mM HEPES, 0.15 M NaCl, pH 7.0, 20 ± 0.2 °C.
Figure 2.18.
Figure 2.18 (cont.)

C

Fluorescence Intensity @ 470 nm

Fast thrombin
Slow thrombin
mzIIa-F1

Iodide (mM)

D

F0/F @ 470

Fast thrombin
Slow thrombin
mzIIa-F1

Iodide (mM)
Figure 2.18 (cont.)

E

F

Fluorescence Intensity @ 520 nm

Iodide (mM)

■ fast thrombin
■ slow thrombin
▲ mzIIa-F1

Iodide (mM)

■ fast thrombin
■ slow thrombin
▲ mzIIa-F1

107
Figure 2.18 (cont.)

G

Fluorescence Intensity @ 520 nm

Iodide (mM)

- fast thrombin
- slow thrombin
- mzIIa-F1

H

F0/F @ 520 nm

Iodide (mM)

- fast thrombin
- slow thrombin
- mzIIa-F1

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### TABLE 2.2

Values of the Stern-Volmer quenching constant ($K_{SV}$) for quenching experiments of $\alpha$-thrombin and mZIIa-F1 with iodide. Buffer was 10 mM Tris, 0.3 M Na⁺ or Ch⁺, 0.3 M Cl⁻ or I⁻, pH 7.0, 20 ± 0.2 °C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{SV1}$ (mM⁻¹)</th>
<th>$K_{SV2}$ (mM⁻¹)</th>
<th>Fraction ($K_{SV1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-thrombin (fast)</td>
<td>0.036</td>
<td>$2.6 \times 10^{-4}$</td>
<td>0.43</td>
</tr>
<tr>
<td>dansyl $\alpha$-thrombin (fast)</td>
<td>0.0023</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>dansyl-EGR-$\alpha$-thrombin (fast)</td>
<td>0.24</td>
<td>$6.0 \times 10^{-4}$</td>
<td>0.12</td>
</tr>
<tr>
<td>$\alpha$-thrombin (slow)</td>
<td>0.015</td>
<td>$4.0 \times 10^{-4}$</td>
<td>0.35</td>
</tr>
<tr>
<td>dansyl $\alpha$-thrombin (slow)</td>
<td>0.0021</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>dansyl-EGR-$\alpha$-thrombin (slow)</td>
<td>1.9</td>
<td>$3.3 \times 10^{-4}$</td>
<td>0.11</td>
</tr>
<tr>
<td>mZIIa-F1</td>
<td>0.049</td>
<td>$1.0 \times 10^{-3}$</td>
<td>0.28</td>
</tr>
<tr>
<td>dansyl mZIIa-F1</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>dansyl-EGR-mZIIa-F1</td>
<td>0.34</td>
<td>$1.0 \times 10^{-3}$</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* Conformation change upon I⁻ binding interfered with quenching
One of the reasons behind mzIIa-F1 (and mzIIa) anticoagulant activity is the fact that these enzymes possess the ability to bind thrombomodulin (TM), which in turn activates the anticoagulation pathway (Doyle and Mann, 1990; Wu et al., 1992; Esmon et al., 1993). The binding of TM alters the active site of α-thrombin as has been shown by ESR and fluorescence (Musci et al., 1988; Ye et al., 1991). Does mzIIa-F1 undergo these same conformational changes? Figure 2.19 answers this question. The titration of dansyl α-thrombin with recombinant TM [TMD1(CS+)] is shown in Figure 2.19A, which is consistent with earlier results from the laboratory of Dr. C. Esmon (Ye et al., 1991). A 30% decrease in dansyl fluorescence was noted upon binding to TMD1(CS+). The results with mzIIa-F1 were quite similar (Figure 2.18B). Again, a 30% decrease in fluorescence was obtained with TMD1(CS+). Extremely tight binding (K_D < 10 nM) was observed for both dansyl-α-thrombin and dansyl-mzIIa-F1. However, while the stoichiometry of binding for α-thrombin is approximately 0.8-0.9, mzIIa-F clearly exhibits a stoichiometry of considerably less (0.5). Possibly, the presence of F2 on the surface of mzIIa-F blocks the binding of the CS moiety to the exosite II. In its place a second mzIIa-F1 binds to the CS in the anion exosite I (Ye et al., 1992). Thus, a 1:2 stoichiometry is observed. These results still suggest that mzIIa-F1 experiences a conformational change similar to that of α-thrombin upon the binding of TM. Also, this study supports other studies that have shown that the anion exosite of mzIIa-F1 (necessary for the binding of fibrinogen and TM) is present and functional in this enzyme (Wu et al., 1994; Ni et al., 1993).
Figure 2.19 Fluorescence titration of dansyl α-thrombin and mzIIa-F1 with TMD1(CS+). Buffer was 40 mM HEPES, 70 mM NaCl, pH 7.0, 15 ± 0.2 °C. Protein concentration was 540 nM. Excitation wavelength was 280 nm and slit widths for both emission and excitation monochromators were 10.0 nm.
2.3.3 Proflavin binding studies of mzlIa-F1.

As discussed earlier, proflavin has been used previously to study the active sites of several serine proteases, including chymotrypsin, trypsin, and α-thrombin (Koehler and Magnusson, 1974). In this study, proflavin was employed to investigate the active site of mzlIa-F1. Figure 2.20 illustrates the binding curves of both α-thrombin and mzlIa-F1. As is clearly shown, proflavin binds α-thrombin much more tightly than mzlIa-F1, with a $K_D$ of 38.0 μM. In fact, the binding for proflavin to mzlIa-F1 is too weak to get a reasonably accurate $K_D$ with this method. It may be possible that mzlIa-F1 does not bind proflavin and the apparently linear increase is due to contaminating α-thrombin in the mzlIa-F1 (typically 1-2 % by SDS-PAGE) For this reason, competitive binding studies with other ligands are quite limited.

The reasons behind the weak binding are not exactly clear. One possible explanation is that the S1 pocket is distorted in mzlIa-F1 and is unable to accommodate the large proflavin molecule. This scenario is unlikely because mzlIa-F1 possesses small substrate activity and small changes in S1 conformation would likely have large consequences on α-thrombin activity and specificity. What is more likely is that the molecule is blocked from entering the pocket by a more constrained active site groove, although not dramatically affecting substrate binding (small substrates). This may also help explain the observation that dansyl fluoride inhibits mzlIa-F1 roughly three times slower than α-thrombin (Figure 2.21).
Figure 2.20 Proflavin binding curves for mzIIa-F1 and α-thrombin. The ordinate is linearly related to the concentration of proflavin bound to enzyme. Buffer conditions are 50 mM Tris, 0.75 M NaCl, pH 6.5, 25 ± 2 °C.
Figure 2.21 Inhibition of α-thrombin and mzIIa-F1 activity by dansyl fluoride. Buffer is 20 mM Tris, 0.15 M NaCl, pH 7.5, 25% isopropanol.
2.4 DISCUSSION

2.4.1 ESR Studies of the conformation of the mzlIa-F1 active site.

The ESR results presented in section 2.3.1 demonstrate that the active site of mzlIa-F1 differs from α-thrombin in the region of the active site in which labels p-IV and p-V probe. However, this conclusion is not extremely useful if the location of this site is not known. Fortunately, the X-ray crystal structures of both p-V and m-V α-thrombin, both displayed in Figure 2.13, have been solved by Nienaber and Berliner (1996). P-V was found to nestle in the fibrinogen binding groove of α-thrombin directly in the S2 subsite. This is the proposed apolar binding site of α-thrombin (Bode et al., 1992). Two isomers (R and S) of each label are shown and spin label studies have typically used racemic mixtures of spin labels. In the case of p-V, there is little difference between the two. This is not the case for m-V, which was found in two loci. The S isomer was found to reside in the S1 specificity pocket, while the R isomer was found to probe the autolysis loop (Trp148). ESR solution studies presumably measure a mixture of the isomers and are generally unable to be resolved into two mobilities. All of the label binding loci are consistent with earlier predictions based on solution studies as well as this work (Berliner and Shen, 1977b; Nienaber and Berliner, 1991).

If the assumption is made that both p-V and m-V reside in the same sites in mzlIa-F1, some conclusions can be made about the mzlIa-F1 active site structure. The m-V data suggests that both the S1 pocket and autolysis loop structures of mzlIa-F1 are comparable to those of α-thrombin. This is consistent with other data, which have shown mzlIa-F1 to have full catalytic activity (binding of the Pl residue is necessary for catalytic activity; Doyle
and Mann, 1990) and a functional anion exosite I (linked to the autolysis loop; Wu et al., 1994). The proposed apolar binding site is necessary for fibrinogen binding, but not small substrates. Thus, if this site is disrupted in mzIIa-F1, the enzyme would retain small substrate activity. This is entirely consistent with the p-V spin label results, and it is likely that the difference in clotting activities of α-thrombin and mzIIa-F1 are due, at least in part, to conformational differences in the S2 - S3 binding sites. This also somewhat supported by a recent mutagenesis study in which an anti-coagulant α-thrombin (mzIIa-F1 like) was engineered by a mutation of Glu$^{229}$ to Ala. This residue is located in the S6 and located 7-9 Å from S3 (Gibbs et al., 1995). The subsites S2-S6 are also relatively close to the F2 noncovalent binding domain (anion exosite II) and could be distorted by the presence of F2. It is interesting, however, that F2 bound non-covalently to α-thrombin does not affect clotting activity (Doyle and Mann, 1990).

2.4.2 Fluorescence and proflavin studies.

Fluorescence studies paint a similar picture of the mzIIa-F1 active site. The drastic differences in properties of dansyl fluoride and dansyl-EGR-CH$_2$Cl labeled α-thrombin and mzIIa-F1 demonstrate that the S4 site of the mzIIa-F1 active site is more hydrophobic and buried. No noticeable differences were noted in fluorescence labels which probe the active site (presumably around S1). However, the fact that proflavin binds mzIIa-F significantly weaker suggests a difference in this region as well. A tempting conclusion is that the loops adjacent to the active site are more restricted in mzIIa-F1. However, the orientation would most likely result in a change in fluorescence emission maximum and intensity. While the
increased an increase in fluorescence intensity is noted for mzIIa-F1, it is impossible to
differentiate whether it is a result of a conformational difference or the increased number of
tryptophans (9 to 11). The crystal structure of the proflavin α-thrombin complex (Figure
1.16) demonstrates that a slight change in conformation of the β-loop (residues 60A-60I; see
Figure 1.3) may be enough to restrict proflavin from entering. It is most likely, therefore,
that the mzIIa-F1 active site groove is more restricted throughout the active site cleft,
however, the most drastic disparity is in the so called apolar binding region (S2-S4).
CHAPTER III

CONFORMATIONAL CHANGES UPON Na⁺ BINDING TO α-THROMBIN

3.1 INTRODUCTION

3.1.1 Goals of the study.

More than 60 enzymes have been found to be activated by monovalent cations (Evans and Sorger, 1966; Suelter, 1970). All but a few of these are activated by K⁺ because the majority reside intracellular where K⁺ is more prevalent than Na⁺. Recently, Wells and Di Cera (1992) reported that α-thrombin specifically binds a Na⁺ ion, resulting in a conformational change which converts α-thrombin to the fast form. A 20% fluorescence increase in fluorescence intensity was reported by Wells and Di Cera (1992). Coupled with this is a large change in heat capacity, which is probably due to a rearrangement of a hydrogen bonded water molecule network in the solvent channel (Guinto and Di Cera, 1996). The consequence of the slow → fast conversion is a relative increase in the acylation rate constant, with all rate constants increasing in the fast form (Wells and Di Cera, 1992).

The goal of this work was to investigate the nature of this conformational change by biophysical methods. As previously mentioned, circular dichroism (CD) and intrinsic fluorescence studies have been shown to be sensitive to the conformational change involved in the slow → fast transition (Villanueva and Perret, 1983; Wells and Di Cera, 1992). From
chemical modification, difference UV, and CD studies, Villanueva and Perret (1983) have concluded that the Na⁺ bound structure is more compact, helical, and that tyrosines, not tryptophans, are involved in the transition. No evidence for any dimerization was found in any form except in the presence of high concentrations of Ca²⁺. Wells and Di Cera (1992) also speculate that the conformational change involves the three active site tryptophans (60D, 148, and 215) and that their data suggest an "opening up" of the loops surrounding the active site, thus making it more accessible to the substrate. This conclusion is contradictory to the Villanueva finding that no tryptophans are involved.

3.2.2 Rationale of the approach and summary of the results.

In this work, ESR active site spin labels were used to probe the active site of slow α-thrombin in a fashion similar to that done earlier with other forms of thrombin (all previous ESR spin label studies have been done under conditions which favor fast α-thrombin). Proflavin absorption difference spectra also reveal information about the slow α-thrombin active site. In addition fluorescence studies on the exposure of tryptophans as well as extrinsic labels provide information about the structure of the slow conformation of α-thrombin.

With the exception of two labels m-V and p-III, the ESR spin labeling resulted in spectra that were more immobilized in the slow form of α-thrombin. This indicates that, with the exception of the S1 specificity pocket (where m-V is known to reside), the active site cleft of slow α-thrombin is more occluded, which is consistent with earlier results by Wells and Di Cera (1992). It is possible, although not probable, that the immobilization may
be due to a change in the aggregation state of the $\alpha$-thrombin molecule (aggregation slows
the tumbling rate of the $\alpha$-thrombin molecule resulting in an immobilized label). However,
the fact that two labels do not experience this immobilization and that earlier studies have
found no aggregation in with slow $\alpha$-thrombin, makes this explanation unlikely (Villanueva
and Perret, 1983).

Proflavin binding studies of slow $\alpha$-thrombin have found that proflavin does not bind
slow $\alpha$-thrombin nearly as tightly as it does fast $\alpha$-thrombin. In fact the binding is too weak
to obtain a reasonable binding constant by proflavin difference absorption. Titrations of Na$^+$
to $\alpha$-thrombin in the presence of proflavin show that an increase in proflavin affinity is
concurrent with Na$^+$ binding. The reason for the lack of binding is most likely steric
occlusion of the proflavin molecule at the top of the S1 pocket, because $m$-V, which also
resides in the S1 pocket, demonstrated little change upon Na$^+$ binding. Also this would be
consistent with the entire active site groove, therefore affecting all labels.

Fluorescence studies of slow and fast $\alpha$-thrombin reveal subtle differences between
the two enzymes. A 20% enhancement in fluorescence intensity was observed by Wells and
Di Cera (1992) upon Na$^+$ binding, suggestive of a conformational change toward less
exposed tryptophans. Acrylamide quenching of slow and fast $\alpha$-thrombin showed little
difference in exposure, but iodide quenching shows fast $\alpha$-thrombin to be more susceptible.
Slow $\alpha$-thrombin is also more stable in the presence of guanidine. Overall, these results are
consistent with a conformational change which results in increased exposure of one
population of tryptophans which is near a positively charged moiety. Perfect candidates for
this are Trp$^{96}$ and Trp$^{237}$. Both are located in the vicinity of anion exosite II and an intriguing
hydrophobic patch. This site is presumably blocked by F2 in mzIIa-F1 and may be the reason that mzIIa-F1 appears to be locked in the slow conformation.

3.2 MATERIALS AND METHODS

3.2.1 Materials.

Choline chloride (lot 102HH5013), tryptamine hydrochloride, 6FT hydrochloride (lot 71110680), crude ECV, and Tris-base were purchased from Sigma Chemical Company. Proflavin hydrochloride (lot 11335) was obtained from Allied Chemical and recrystallized in ethanol prior to use. Amberlite CG-50 was purchased from Pharmacia, Inc. S-2238, chromothrombin, and CBS 34.47 were purchased from Kabi and Diagnostica. Spin labels were previously synthesized according to Wong et al. (1974). All other chemicals were of at least reagent grade and used without further purification.

3.2.2 Proteins.

3.2.2.1 Activation of human prothrombin to α-thrombin.

Human prothrombin was obtained as a gift from Dr. Dan Walz (Wayne State University, Detroit, MI). The prothrombin was stored in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.0, and was activated as such with 1 mg of crude Echis carinatus venom (ECV) per 100 mL of prothrombin. The reaction was monitored for amidolytic activity while incubating at room temperature for 2.5 hours. When the reaction had proceeded to completion, the sample was adjusted to pH 7.8 with 1 M Tris, pH 7.8 and applied to a column of Amberlite CG-50 (3 cm X 30 cm). The column was then washed with 0.15 M
NaCl (unbuffered) and the α-thrombin was eluted with unbuffered 0.75 M NaCl. The fractions were then analyzed for absorbance at 280 nm and amidolytic activity, with the fractions containing greater than 0.05 mg/ml stored at -60°C. All human α-thrombin used in this study was from the same lot (PDB01) and the properties of the fractions are given in Table 3.1.

3.2.3 ESR Spectroscopy.

3.2.3.1 Spin labeling.

Human α-thrombin was spin labeled with p-I, p-III, p-V, m-IV, m-V, or m-VII according to the procedure described in section 2.2.4.1 When "slow" α-thrombin was desired, samples were dialyzed vs. buffer containing the appropriate concentration of choline (0.3 M, 0.5 M, or 0.75 M) to maintain equal ionic strength as those experiments performed in NaCl.

3.2.3.2 ESR Spectra.

ESR spectroscopic measurements were performed as described in section 2.2.4.2, except that dialysis was performed vs. 10 mM Tris, 0.3 M choline or sodium chloride, pH 6.5.

3.2.4 Fluorescence Spectroscopy.

3.2.4.1 Fluorescence labeling.

Dansyl-EGR-CH₂Cl labeling of α-thrombin was performed as described in section
TABLE 3.1

Activities of α-thrombin fractions used in this study. Purification and clotting assays are described in sections 3.2.2.1 and 3.2.6.2, respectively. Active site titration was done by NPGB assay and is described in section 3.2.6.3. A (---) indicates that the assay was not performed.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration (mg/ml)</th>
<th>Clotting Activity (U/ml)</th>
<th>Active Site Titration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.63</td>
<td>1349</td>
<td>120</td>
</tr>
<tr>
<td>21</td>
<td>3.32</td>
<td>3012</td>
<td>107</td>
</tr>
<tr>
<td>22</td>
<td>3.83</td>
<td>3002</td>
<td>105</td>
</tr>
<tr>
<td>23</td>
<td>2.17</td>
<td>3133</td>
<td>112</td>
</tr>
<tr>
<td>24</td>
<td>0.83</td>
<td>4016</td>
<td>75.1</td>
</tr>
<tr>
<td>25</td>
<td>0.4</td>
<td>4250</td>
<td>71.9</td>
</tr>
<tr>
<td>38</td>
<td>2.61</td>
<td>2452</td>
<td>---</td>
</tr>
<tr>
<td>39</td>
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<td>2234</td>
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<td>7.16</td>
<td>2234</td>
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<td>7.16</td>
<td>2234</td>
<td>---</td>
</tr>
<tr>
<td>42</td>
<td>5.46</td>
<td>2014</td>
<td>---</td>
</tr>
<tr>
<td>43</td>
<td>2.32</td>
<td>1724</td>
<td>---</td>
</tr>
<tr>
<td>44</td>
<td>1.31</td>
<td>1183</td>
<td>---</td>
</tr>
<tr>
<td>45</td>
<td>0.70</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
2.2.5.1. Bis-ANS was covalently labeled to α-thrombin by incubating 10-80 μM α-thrombin with 30-600 μM bis-ANS in 20 mM Tris, 0.15 M NaCl, pH 7.0. The sample was aliquoted into a 96-well plate and incubated on ice for 10 min. Then the samples were irradiated with UV light from a hand held UV lamp (Ultraviolet Products UVS-11, 0.16 A) with no filter at a distance of 1 cm for 1 h. These samples were then dialyzed exhaustively vs. 50 mM Tris, 0.75 M NaCl, pH 6.5 to remove any free label. This sample was then diluted into the appropriate buffer to a concentration of approximately 0.5 μM for fluorescence measurements.

3.2.4.2 Fluorescence Spectroscopy.

Fluorescence measurements were obtained as described in section 2.2.5.2.

3.2.4.3 Fluorescence quenching studies

Quenching experiments were performed as described in section 2.2.5.3.

3.2.5 Proflavin binding.

Proflavin binding experiments were performed as described in section 2.2.6. Sodium titrations were performed by the replacement of 20 mM Tris, 0.15 M choline chloride, pH 7.0 with 20 mM Tris, 0.15 M NaCl, pH 7.0, each buffer containing identical concentrations of proflavin and α-thrombin, as well as constant ionic strength. Proflavin inhibition experiments are described in section 3.2.6.1.
3.2.6 Activity Assays.

3.2.6.1 Amidolytic assays

Activity assays were generally performed as described in section 2.2.7.1. The buffer contained the appropriate concentration of sodium ions or effector, keeping care to keep a constant ionic strength. The effector generally could not be added as a stock solution due to limiting solubility.

3.2.6.2 Clotting assays

Clotting times were determined on a BRL fibrometer. Buffer was 10 mM imidazole, 0.67 mg/mL PEG 6000, 0.1 M NaCl, 2.0 mM CaCl₂, pH 7.4. Fibrinogen concentration was 8 mg/mL. Human fibrinogen was incubated at 37°C with enough α-thrombin to clot in approximately 20 s. This clotting time was compared to a standard curve prepared using NIH standard α-thrombin to calculate the number of NIH units/mg of α-thrombin.

3.2.6.3 Active site assay

The NPGB (p-nitrophenyl-p-4'-guanidinobenzoate) assay was performed to determine the number of active sites available on α-thrombin. Both sample and reference cuvettes contain 1000 μL of 50 mM barbital, 0.15 M NaCl, pH 8.3 and 5 μL of 10 mM NPGB in 1:4 DMF:acetonitrile. α-Thrombin concentrations range from 1 to 7 μM. Once α-thrombin was added to the sample cuvette, a burst phase and acylation phase are observed while monitoring the absorbance at 410 nm. The acylation was then extrapolated back to time zero. This absorbance was used to calculate the concentration of active sites.
3.3 RESULTS

3.3.1 ESR active site comparison.

In order to learn more about the active site environment of slow α-thrombin (all previous ESR studies have been performed under conditions in which the fast form is predominant), the ESR spectra of spin labeled human α-thrombin were recorded under fast and slow conditions (0.3 M NaCl and 0.3 M choline chloride, respectively). The resulting 2T₁ values are given in Table 3.2 with the spectra provided in Figures 3.1 and 3.2. Although label mobilities increase in general upon Na⁺ binding, the data show no obvious differences in the motion of meta and para labels, as seen with other ligand such as indole and benzamidine. However, if just the labels in which crystal structures are available (m-V and p-V) are considered, predictions of the slow structure can be made. From the X-ray crystal structures, the label m-V was found to probe both the S1 pocket and the autolysis loop (Nienaber and Berliner, 1996). Since little change was noted between the slow and fast form, it would be reasonable to predict that only subtle differences in structure are present in this region of the slow α-thrombin active site. This result is in agreement with Landis et al. (1981) who concluded that no conformational change, detectable by ESR, occurs upon Na⁺ binding. However, results from only m-V were reported.

On the other hand, p-V was found to lie in the fibrinogen binding groove near S2 and S3 in the opposite direction of m-V (Nienaber and Berliner, 1996). The large differences in p-V mobilities indicate conformational or structural differences between slow and fast α-thrombin. Thus, it can be concluded that the slow→fast transition involves a conformational change in the S2 or S3 region of the active site, the same region which was found to differ
Figure 3.1 X-band ESR spectra of m-IV, m-V, and m-VII labeled human α-thrombin in either the fast or slow form. Buffer is 20 mM Tris, 0.3 M choline or sodium chloride, pH 6.5.

(a) m-IV α-thrombin in 0.3 M choline chloride
(b) m-IV α-thrombin in 0.3 M NaCl
(c) m-V α-thrombin in 0.3 M choline chloride
(d) m-V α-thrombin in 0.3 M NaCl
(e) m-VII α-thrombin in 0.3 M choline chloride
(f) m-VII α-thrombin in 0.3 M NaCl
Figure 3.1.
Figure 3.1. (cont.)

\[ \begin{align*}
20 \text{ Gauss} \\
\text{e)} \\
\text{f)} \\
\text{m-VII} \\
(\text{m-NCO-6NH})
\end{align*} \]
Figure 3.2  X-band ESR spectra of p-III, p-IV, and p-V labeled human α-thrombin in slow or fast conditions. Buffer was 20 mM Tris, 0.3 M choline chloride or 0.3 M NaCl, pH 6.5. α-Thrombin concentration was 50-60 μM.

(a) p-III α-thrombin in 0.3 M choline chloride
(b) p-III α-thrombin in 0.3 M NaCl
(c) p-IV α-thrombin in 0.3 M choline chloride
(d) p-IV α-thrombin in 0.3 M NaCl
(e) p-V α-thrombin in 0.3 M choline chloride
(f) p-V α-thrombin in 0.3 M NaCl
Figure 3.2.
Figure 3.2. (cont.)

20 Gauss

\[ \text{SO}_2 \text{F} \]

\[ \text{C}=\text{O} \]

\[ \text{H}-\text{N} \]

\[ \text{p-V} \]

(p-CO-5NH)
in the mzIIa-F1 structure (Chapter II), providing support for Di Cera's prediction that mzIIa-
F1 is locked into the slow form (Dang et al., 1995).

Of the other labels, the unexpected result comes from m-IV and m-VII, which both exhibit a large immobilization in the slow form in comparison with the fast. Thus, it is possible, due to minor conformational differences, that m-IV and m-VII do not reside within the pocket as m-V was found in the crystal structure (Figure 2.13), therefore giving rise to a large change upon the slow to fast conversion. It is reasonable to conclude that for m-VII the deviation from other meta labels is due to the label being longer than m-V and thus unable to fit in the pocket. However, the only difference between m-IV and m-V is the size of the nitroxide ring. This striking difference in label properties is interesting, but not unprecedented (Boxrud and Berliner, 1996). Clearly, minute differences in label structure may have large consequences on where the label is able to bind, thus exhibit drastic differences in label mobility. All the other labels, except p-III exhibited a large change upon Na+ binding. This is not surprising since they have been previously found to probe the same region of the active site as p-V, reported as the indole site (Berliner and Shen, 1977b; Nienaber and Berliner, 1991; Nienaber and Berliner, 1996).

3.3.2 Proflavin studies of slow and fast α-thrombin.

As with mzIIa-F1 (Chapter II), proflavin binding studies were performed on slow α-
thrombin. Figure 3.3 shows a proflavin binding curve of human slow and fast α-thrombin. Slow α-thrombin was found to exhibit very weak binding to proflavin, and as with mzIIa-F1, a reasonable Kd was impossible to obtain from this experiment. Again, this precludes
TABLE 3.2

2T₁ values (± 0.5 G) obtained from the spectra of spin labeled human α-thrombin in the slow and fast forms. The buffer in these studies was 10 mM Tris, 0.3 M NaCl or 0.3 M choline chloride, pH 6.5.

<table>
<thead>
<tr>
<th>Label</th>
<th>Fast form</th>
<th>Slow form</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-IV</td>
<td>46.0 G</td>
<td>58.5 G</td>
</tr>
<tr>
<td>m-V</td>
<td>60.2</td>
<td>59.5</td>
</tr>
<tr>
<td>m-VII</td>
<td>47.5</td>
<td>61.0</td>
</tr>
<tr>
<td>p-I</td>
<td>59.7</td>
<td>62.4</td>
</tr>
<tr>
<td>p-III</td>
<td>&lt; 45</td>
<td>&lt; 45</td>
</tr>
<tr>
<td>p-V</td>
<td>53</td>
<td>60.2</td>
</tr>
</tbody>
</table>
proflavin as a probe of other ligands of slow α-thrombin, but does reveal something about the slow α-thrombin active site. As with mzIIa-F1, the active site groove is less accessible to substrates and thus sterically occludes proflavin from binding the active site. This hypothesis was also postulated by Wells and Di Cera (1992), based on the changes in fluorescence properties.

Based on the fact that proflavin binds fast α-thrombin selectively, proflavin can be employed to monitor the slow → fast transition. Figure 3.4 shows a titration of both human α-thrombin and bovine mzIIa-F1 with Na⁺ (ionic strength held constant with choline chloride). Note that α-thrombin exhibits a hyperbolic binding curve with a $K_D$ of 19.1 mM, which is consistent with values reported by Wells and Di Cera (1992). On the other hand, mzIIa-F1 shows no Na⁺ binding (at least no effect upon proflavin binding) and appears to be "locked into" a conformation similar to slow α-thrombin. This again, is consistent with Di Cera's hypothesis that mzIIa-F1 is a permanent slow form of α-thrombin (Dang et al., 1995).

### 3.3.3 Fluorescence studies of slow and fast α-thrombin.

To better answer the question of what is happening to the α-thrombin conformation during the slow → fast transition, the exposure of tryptophans to acrylamide quenching was evaluated. The Stern-Volmer quenching curves of slow and fast α-thrombin intrinsic fluorescence are presented in Figure 3.5. From this data, the exposure of tryptophans is equal within experimental error with obtained $K_{SV}$ values of 0.01276 M⁻¹ and 0.0118 M⁻¹ for slow and fast α-thrombin, respectively. Because no dramatic change in tryptophan environment was noted, the data in this work support the hypothesis that tyrosine residues are more
Figure 3.3 Proflavin binding curve of α-thrombin under slow and fast conditions.

The ordinate is the peak to trough (468 and 440 nm, respectively) height of the difference absorption spectra of proflavin bound minus free proflavin. Buffer was 20 mM Tris, 0.15 M NaCl, pH 7.0 for fast α-thrombin and 0.15 M choline chloride replacing NaCl in the slow form. Proflavin concentration was 0.8 µM.
Figure 3.4 Sodium binding isotherm of the proflavin-α-thrombin and the proflavin-mzIIa-F1 complexes.

α-Thrombin, mzIIa-F1, and proflavin concentrations were 20, 20, and 0.8 μM, respectively. Initial buffer was 20 mM Tris, 0.15 M choline chloride, pH 7.0 and titrating buffer contained enzyme, proflavin and 0.15 M NaCl replacing choline chloride.
Figure 3.4.

proflavin
instrumental in the conformational change than are tryptophans. Since slow α-thrombin plays a vital role in hemostasis, it would be prudent to investigate its structure more thoroughly. Figure 3.6 shows a comparison of the stability of slow and fast α-thrombin to unfolding by guanidine monitored by intrinsic fluorescence. As has been the case in all studies of the two enzymes, the differences between the two are quite subtle. The fast form denatures at approximately 0.5 M earlier than the slow form. This result is somewhat surprising since Na⁺ binding is generally found to stabilize protein structures (Suelter, 1970). This result is rational if one considers the role of α-thrombin. In the event of vascular injury, α-thrombin activity must be very short lived to prevent excess clotting which could injure or kill the organism. α-Thrombin is therefore tightly regulated. If the fast form is more structurally unstable, it is more susceptible to thermal denaturation and proteolytic degradation. It would be advantageous for fast α-thrombin to be removed as quickly as possible, while slow α-thrombin is vital for the anticoagulation pathway. More studies would be interesting to determine if in fact fast α-thrombin is cleared more quickly than slow α-thrombin in vivo.

The reversible fluorescent probe bis-ANS, which binds non-specifically to hydrophobic sites, was also employed to investigate the slow → fast transition. Titration of human α-thrombin in sodium concentrations of 0, 0.1, 0.2 and 0.3 M are shown in Figure 3.7A. As is clear from the curves, several binding sites for bis-ANS exist. A strong site with a $K_D$ of 4.1 μM is present for each of the α-thrombin conditions. For the fast form, an additional bis-ANS site is exposed with a $K_D$ of about 15-20 μM. The appearance of an additional hydrophobic site facilitates another tool for following the slow
Figure 3.5 Stem-Volmer plot of acrylamide quenching of slow and fast α-thrombin.

Protein concentration was 1.0 μM. Buffer was 20 mM Tris, 0.15 M choline chloride or NaCl, pH 7.0, 20 ± 0.2 °C. Excitation wavelength and slit widths were 280 and 6.0 nm, respectively.
Figure 3.6 Denaturation curves of slow and fast $\alpha$-thrombin with guanidine as measured by intrinsic fluorescence. Protein concentration was 1.0 $\mu$M. Excitation and emission wavelengths were 280 and 340 nm, respectively. Slit widths were 6.0 nm. Buffers were identical to those in Figure 3.5.
Figure 3.7 Titration of A. human α-thrombin and B. bovine mzIIa-F1 with bis-ANS in several Na⁺ concentrations.

Protein concentration was 0.5 μM. Buffers were 20 mM Tris, 0.3 M (choline + Na⁺) chloride, pH 7.0, 15 ± 0.2 °C. Excitation and emission wavelengths were 390 and 490 nm respectively. Slit widths were 8.0 nm.
Figure 3.7.
fast transition. Figure 3.8 shows a titration with Na' of bis-ANS \( \alpha \)-thrombin in choline, with a \( K_D \) of 4.8 mM was obtained from this curve. This value is somewhat surprising and illustrates the danger of measurement of binding using only one method. The binding constant measured by bis-ANS fluorescence is approximately 4-fold lower than is expected (and was measured by activity, proflavin binding, and intrinsic fluorescence). It is entirely possible that bis-ANS, since it is negatively charged, induces the binding of Na' and enhances the binding constant. However, this is somewhat unlikely because the Na' site of \( \alpha \)-thrombin is somewhat buried and is most likely inaccessible to bis-ANS (Di Cera, 1995). Another intriguing possibility is the possibility that the bis-ANS site is thermodynamically linked with the Na' and occupation of this site enhances Na' binding. Obviously, for this result to be physiologically significant, a ligand which is present in plasma must occupy the site. Clearly more work in this area is necessary.

The question of whether mzIIa-F1 possesses the above discussed hydrophobic site is answered in Figure 3.7B. This shows that up to 200 mM NaCl has no effect on this second site, and in fact, mzIIa-F1 behaves as slow \( \alpha \)-thrombin. This again corroborates earlier data that mzIIa-F1 is indeed locked into the slow form. The 300 mM NaCl titration, exhibits an increase in bis-ANS fluorescence intensity. However, rather than being due to the appearance of a second site, it appears to be consistent with an increase in quantum yield of the bis-ANS. The most likely reason for this is a charge effect of Na' non-specifically adsorbed to the \( \alpha \)-thrombin surface, resulting in a conformational difference of the bound bis-ANS to a more hydrophobic environment.
Figure 3.8 Titration of bis-ANS labeled α-thrombin with Na⁺.

Protein and bis-ANS concentrations were 20 μM and 0.5 μM, respectively. Buffers were identical to those in Figure 3.4. Fluorescence parameters were identical to those in Figure 3.7.
3.4 DISCUSSION

3.4.1 Active site ESR studies.

From the ESR spectra of human α-thrombin labeled with m-IV, m-V, m-VII, p-I, p-III, or p-V, it is clear that the binding of Na⁺ affects the conformation of the α-thrombin active site. It should also be noted that of the labels which did show large differences (m-IV, m-VII, p-I, and p-V), all show an increase in mobility upon the binding of Na⁺. Aggregation of slow α-thrombin molecules would likely cause an immobilization of spin labeled α-thrombin, as well as a conformational difference. Because of this, the possibility of aggregation must be ruled out. Aggregation is unlikely because, with the exception of Ca²⁺, Villanueva and Perret (1983) found no evidence of dimerization by sedimentation centrifugation experiments. Also, aggregation would likely result in an immobilization of all labels since macroscopic tumbling rates would be slowed. Thus, the p-III and m-V results suggest that aggregation does not occur. Finally, the surface of slow α-thrombin was found to be less hydrophobic by bis-ANS fluorescence making aggregation of slow α-thrombin more unlikely. There are two potential reasons for the immobilization observed upon Na⁺ binding. One is that the active site of slow α-thrombin is more hydrophobic, thus making the surface more "sticky," providing a surface for the hydrophobic nitroxide ring to adhere. This interpretation is not likely because the slow form was found to be less hydrophobic than the fast form by the bis-ANS fluorescence studies and proflavin, a hydrophobic ligand, was found to bind the fast form more tightly. A second, more likely, hypothesis is that the immobilization is due to steric occlusion of the label. Wells and Di Cera (1992) also present supporting evidence for constriction of the fibrinogen binding groove in slow α-thrombin.
3.4.2 Fluorescence studies of the slow $\rightarrow$ fast transition.

The finding that the tryptophans of slow and fast $\alpha$-thrombins are roughly equal in their exposure is not altogether surprising considering the intrinsic fluorescence differences are not substantial (Wells and Di Cera, 1992). However, it does raise the question of what conformational change is occurring in the slow $\rightarrow$ fast transition. Studies by Villanueva and later by Di Cera appear to be contradictory (Villanueva and Perret, 1983; Wells and Di Cera, 1992). The conclusion of Villanueva and Perret (1983), based on CD, difference UV, and chemical modification studies, was that tyrosines were responsible for the conformational differences. As stated earlier in this chapter, Wells and Di Cera concluded that the loops surrounding the active site closed in on the active site groove. However, this would undoubtedly affect the environment of three critical tryptophans (60D, 148, 215) and even a slight shift in the loop conformation would result in large fluorescence changes, changes in tryptophan exposure, and sensitivity to chemical modification. It is possible that one of the tyrosines that Villanueva and Perret detected in their work was Tyr$^{224}$. This has been implicated by Di Cera recently as part of the Na$^+$ binding site (specifically the backbone carbonyl oxygen), and was found to be extremely important in determining whether serine proteases are Na$^+$ activated (Di Cera et al., 1995; Di Cera, personal communication). It is likely that this residue undergoes a conformational change upon Na$^+$ binding. There are also two tyrosines around in the active site which could also be involved (Bode et al., 1992). Tyr$^{60A}$ makes up the S2 binding domain which has been implicated earlier in this chapter as experiencing large changes in ESR spin label environments. A small conformational change in this site would certainly affect S2 as well as the entire beta loop, which may be responsible
for the proflavin exclusion. A second tyrosine, Tyr228 lines the S1 specificity pocket and could have drastic effects on the conformation of this pocket.

The fact that fast α-thrombin is more hydrophobic in nature (at least presents its hydrophobic surface in a more ordered orientation) than slow is an interesting one. Several implications present themselves. The most obvious of these is possible interactions with apolar ligands such as serotonin, tryptamine and ATP which are secreted by activated platelets during clotting. Interactions of slow (and fast) α-thrombin will be discussed in further detail in chapter 4. A more hydrophobic surface of α-thrombin may also promote non-specific membrane interactions, which could effectively remove α-thrombin from the clotting pathway. Finally, exposure of hydrophobic residues to the surface would also contribute to instability of the α-thrombin structure, thus, helping to explain the earlier denaturation studies in which fast α-thrombin was found to denature at lower guanidine concentrations.

Calculations by Guinto and Di Cera (1996) have shown that the large ΔG associated with the slow → fast transition is due in large part to the extensive network of hydrogen bonded water molecules present in the solvent channel which must reorient upon Na+ binding. If this ΔG were due to only changes in the hydrophobic surface, the conformational change would cover over 50 % of the surface of the protein. This is obviously not the case, but these authors do not rule out some hydrophobic interactions. Thus, it is possible that the large ΔG may be due in part to changes in hydrophobicity of the α-thrombin surface.
CHAPTER IV
APOLAR LIGANDS OF $\alpha$-THROMBIN

4.1 INTRODUCTION

4.1.1 Goals of the study.

Upon activation by $\alpha$-thrombin, platelets secrete a number of compounds into the plasma, of which the most common are serotonin, $\text{Ca}^{2+}$, ATP, and ADP (Detweiler and Wasiewski, 1977). The interaction of these molecules with $\alpha$-thrombin has been found to be quite complex and interesting (Berliner and Shen, 1977b; Conery and Berliner, 1983; Berliner et al., 1986). Evidence for multiple binding sites was found for serotonin, ATP, and tryptamine (Conery and Berliner, 1983 Berliner et al., 1986). Indole and tryptamine were found to activate esterase and clotting activities. The effect of these interactions, if any, on the physiology of $\alpha$-thrombin is largely still unknown.

The goal of this work is to obtain a better understanding of the physiological interactions between $\alpha$-thrombin and these apolar compounds. The differences between slow and fast $\alpha$-thrombin also play a role in these studies. The large heat capacity change in the slow to fast transition may be due in part to a change in surface hydrophobicity, which would certainly have implications for apolar ligand binding (Wells and Di Cera, 1992; Guinto and Di Cera, 1996). The possibility exists that one or more apolar binding sites may
appear or disappear in the slow → fast transition and the interaction with apolar molecules secreted by platelets may differ significantly.

4.1.2 Rationale of the approach and summary of the results.

ESR and activity studies have been successfully used in the past to study the binding of apolar ligands to α-thrombin and its derivatives (Berliner and Shen, 1977a; Conery and Berliner, 1983). In this study, ESR, fluorescence and activity assays are utilized in the study of the interaction of apolar ligands with both slow and fast human α-thrombin. Limited solubility, weak binding, and strong ligand absorption at strategic wavelengths complicate these studies, such that a variety of techniques are necessary.

Spectra of spin labeled human α-thrombin under slow conditions in the presence of apolar ligands reveal that the effects of these ligands on slow α-thrombin are somewhat different than those on fast α-thrombin. In nearly every case, an immobilization was observed in the presence of the apolar ligands indole, proflavin, tryptamine, or 6-fluorotryptamine. The largest immobilization observed was 3.3 G in the case of proflavin binding to m-V labeled α-thrombin. Indole was found to dramatically increase the deacylation rates as well as the immobilization of both p-I and p-III labeled α-thrombin. It is clear from this data that all four of these apolar ligands bind to slow α-thrombin and effect changes in the active site conformation. The conformational effect of thrombin receptor peptide were also observed. This ligand is believed to facilitate the slow → fast conversion (De Cristofaro et al., 1995). Instead of inducing a mobilization of the labels as expected, an immobilization was noted. This may be due to an artifact such as dimerization (some
evidence exists for dimerization around anion exosite binding peptides; Rowand, 1990) or a conformational change in the active site which is different from that of the slow → fast transition.

Kinetic studies of tryptamine and 6-fluorotryptamine inhibition of α-thrombin under slow and fast conditions reveal very different results. Under fast conditions, both tryptamine and 6-fluorotryptamine inhibit α-thrombin competitively with $K_i$'s of 26 mM and 11 mM respectively. However, under slow conditions, both ligands effect an activation of α-thrombin activity with a $K_i$ of approximately 1.5 mM followed by weak inhibition ($K_i > 50$ mM). This is indicative of weaker binding to the S1 pocket, as seen before with mzIIa-F1 and slow α-thrombin (Chapters II and III). The activation is thought to be due to the binding of a second molecule of tryptamine or 6-fluorotryptamine to α-thrombin, which increases the deacylation rate of hydrolysis of substrate. Because deacylation is rate limiting in slow α-thrombin and not fast α-thrombin, an apparent rate enhancement is observed. Studies of mzIIa-F1 show that no activation is present in mzIIa-F1, again suggesting that this site is occluded in this enzyme. Also, weak binding to the S1 pocket was observed; as found previously (Chapter II).

Fluorescence studies using the probe bis-ANS photo-incorporated into α-thrombin show that the fluorescence properties of this new covalent species are similar to those of the non-covalent complex, with some notable exceptions. The emission maxima for labeled slow and fast α-thrombin are quite blue-shifted (13 nm for slow conditions and 26 nm for fast) from that of the non-covalent complex. Also the difference between slow and fast α-thrombin is much more pronounced ($\lambda_{max}$ for slow is 478 nm compared to 466 nm for fast).
The most surprising interaction is one that did not occur. The protonated amino group of 6FT was thought to form a salt bridge with Asp$^{189}$. This, however, is not the case. In fact, the amino moiety of 6FT appears to be unprotonated and forms hydrogen bonds with the main chain carbonyl oxygens of Gly$^{197}$, Val$^{213}$, and Gly$^{216}$. The nitrogen of the indole ring forms a hydrogen bond with the backbone of Gly$^{218}$. This crystal structure was solved by Dr. V. L. Nienaber at DuPont-Merck by soaking crystals of α-thrombin (inhibited with hirudin C-terminal peptide) with 0.5 mg/mL 6FT (Boxrud et al., 1996).
Figure 4.1.
This is also evident in the Na\textsuperscript{+} titration in which similar binding constants were obtained 6.1 vs. 4.3 mM (both have been found to enhance Na\textsuperscript{+} binding to \(\alpha\)-thrombin), but the non-covalent complex resulted in an enhancement of fluorescence while the covalent revealed a quenching. For other ligands such as ATP, thrombomodulin and hirudin, the covalent complex was quite similar in nature to the non-covalent one.

4.2 MATERIALS AND METHODS

4.2.1 Materials.

Choline chloride (lot 102H5013), tryptamine hydrochloride, 6-fluorotryptamine hydrochloride (6FT, lot 71H0680), indole (lot 36C0204), serotonin, and Tris-base were purchased from Sigma Chemical Company. Bis-ANS (lots 45415 and 9C-1) was purchased from Molecular Probes, Inc. Chromogenic TH and CBS 34.47 were purchased from Diagnostica Stago, respectively. Ammonium persulfate, TEMED, Coomassie brilliant blue, and SDS were purchased from Bio-Rad. Trypsin (lot TRL2HX) was obtained from Worthington Enzymes. All other chemicals were of at least reagent grade and used without further purification.

4.2.2 Proteins.

4.2.2.1 Activation of human prothrombin to \(\alpha\)-thrombin.

Activation of human prothrombin is described in section 3.2.2.1.
4.2.3 Electrophoresis.

Electrophoresis was performed as described in section 2.2.3. Bis-ANS α-thrombin was visualized by illuminating the gels (no staining) with UV light and photographing. These gels were dried as described earlier. Occasionally, negative staining was used to enhance contrast. This was accomplished by washing the gels in water for 30 min., incubating in 0.2 M imidazole, 0.1% SDS for 15 min, followed by 0.2 M ZnSO₄ for 30-60 s. The gels were transferred to distilled water when staining was appropriate.

4.2.4 ESR Spectroscopy.

4.2.4.1 Spin labeling.

Spin labeling procedures are described in section 2.2.4.1.

4.2.4.2 ESR Spectra.

ESR spectroscopy measurements are described in section 2.2.4.2.

4.2.5 Proflavin binding.

Proflavin experiments are described in section 2.2.6. Proflavin inhibition measurements are described in section 3.2.6.1.

4.2.6 Activity Assays.

4.2.6.1 Amidolytic Assays

Chromogenic assays are described in section 3.2.6.1.
4.2.6.3 Clotting assay of α-thrombin and meizothrombin (desF1).

Clotting assays are described in section 2.2.7.3.

4.3 RESULTS

4.3.1 Kinetic studies of tryptamine and 6-fluorotryptamine on slow and fast α-thrombin.

As discussed earlier in Chapter I, the interactions of aromatic molecules, such as indole, as well as molecules containing aromatic moieties including tryptamine, serotonin (5-hydroxy-tryptamine) and ATP with α-thrombin are quite complex. Conery and Berliner (1983) found that salt concentration exhibits an effect on the activation of α-thrombin esterase activity by some apolar ligands (indole, tryptamine, and 6-fluorotryptamine (6FT)), with the activation being more pronounced in low salt. With the recent discovery by Wells and Di Cera (1992) that α-thrombin exists in two conformations, it is probable that apolar ligands interact differently with slow and fast α-thrombin, especially in light of the differences in hydrophobicity (Chapter III). The effect of tryptamine on amidolytic activity of α-thrombin in several sodium concentrations (ionic strength is kept at 0.3 M with choline chloride) is shown in Figure 4.2A. Note that at high sodium concentrations (above 30 mM NaCl) tryptamine exhibits simple competitive inhibition of α-thrombin with an inhibition constant (K_i) of 26.4 mM. This finding is consistent with both earlier inhibition studies, in which simple competitive inhibition was observed, and crystal structures of α-thrombin complexed with tryptamine based inhibitors, which were found to bind the S1 specificity pocket (Casale et al., 1995; Boxrud et al., 1996). In the absence of Na^+, on the other hand,
Figure 4.2 The kinetics of tryptamine interactions with slow and fast α-thrombin. A. Inhibition of α-thrombin chromogenic TH activity by tryptamine in increasing Na⁺ at constant ionic strength. B. The influence of tryptamine on the activation of α-thrombin by Na⁺. The buffer in both cases was 10 mM Tris, 0.5 mg/mL PEG6000, pH 8.1 with a total choline and sodium concentration of 0.3 M. The curves presented are apparent binding isotherms of Na⁺ based on activation of α-thrombin. Substrate was 200 μM.
Figure 4.2.
the kinetics are somewhat different. The sigmoidal inhibition curve is indicative of two binding sites for tryptamine. Consistent with the proflavin studies of Chapter III, tryptamine inhibition is much weaker in slow α-thrombin. In addition, a tighter binding \( (K_D \approx 2 \text{ mM}) \) activation site becomes apparent, which is in agreement with previous clotting studies (Berliner et al., 1986).

The effect of tryptamine on Na⁺ activation of α-thrombin is shown in Figure 4.2B. Clearly, the binding of tryptamine eliminates this activation. There exist two possible explanations for this. One is that the binding of tryptamine to α-thrombin sterically occludes Na⁺ from binding, thus inhibiting activation and locking α-thrombin into the slow form. A second is that tryptamine and Na⁺ bind separate sites which are thermodynamically linked. Although there exist some hints that 6-fluorotryptamine may bind in the solvent channel in which the Na⁺ is located (This will be discussed further in section 4.4; Boxrud et al., 1996), the latter possibility is more likely. In this scenario, both tryptamine and Na⁺ facilitate the slow → fast transition. Thus, the binding of one site influences the other to bind more tightly, as is seen in the case of TM (De Cristofaro et al., 1995; Di Cera et al., 1996).

The results with 6FT replacing tryptamine are shown in Figure 4.3. 6FT, while not being significant, was chosen for these studies because of the large activations exhibited in earlier clotting studies (Conery and Berliner, 1983; Berliner et al., 1986). The enhancement of 6FT over tryptamine was thought to be due to the increased hydrophobicity of 6FT. The results in Figure 4.3, although very similar to tryptamine reflect this hydrophobic nature. Again, in the presence of Na⁺ ions competitive inhibition was observed. An inhibition constant of 11.3 mM reflects tighter binding of the more hydrophobic inhibitor. Also, the
extent of activation was greater than that seen for tryptamine, similar to the results seen with clotting (Berliner et al., 1986). The activation of amidolytic substrate activity had not been previously observed, except in the case of fibrinogen. Figure 4.4 shows the progress curves and double reciprocal plots of 6FT "inhibition" of α-thrombin under slow conditions. The Michaelis-Menton parameters for this activation are shown in Table 4.1. Note that an increase in $V_{\text{max}}$ and apparent $K_m$ is observed upon increasing 6FT, indicating mixed inhibition of α-thrombin. It is for this reason that this activation is only seen at high substrate concentrations (negating the effect of increasing $K_m$).

The influence of Na$^+$ on tryptamine inhibition of mzIIa-F1 paints a different picture (Figure 4.5). Even in the absence of Na$^+$, inhibition of mzIIa-F1 fits simple competitive inhibition models. Inhibition by 6FT is increased in the presence of increasing Na$^+$ concentrations, approaching 200 mM. Thus, the 6FT activation site is blocked in mzIIa-F1 as observed for indole and esterase activity (Figure 2.15). The influence of Na$^+$ on mzIIa-F1 activity is also quite interesting. One would anticipate if mzIIa-F1 was locked into a slow state, that Na$^+$ would not influence mzIIa-F1 activity. On the contrary, the Na$^+$ influence on mzIIa-F1 appears to be biphasic. An initial inhibition appears at a $K_i$ of approximately 15 mM (coincidentally near that of α-thrombin activation) followed by an activation at high NaCl concentrations (> 200 mM). The inhibition phase is unaffected by tryptamine presence, but the activation is eliminated by increasing tryptamine, as it is with human α-thrombin. This suggests that the Na$^+$ site in mzIIa-F1 may be functional, but weaker than that of α-thrombin and that an additional Na$^+$ inhibitory site is present on mzIIa-F1. If this is indeed the case, the most logical location for the inhibitory site is on F2 or its interface.
Figure 4.3 Kinetics of 6-fluorotryptamine (6FT) on slow and fast α-thrombin. A. The influence of Na⁺ on the inhibition of α-thrombin chromogenic TH activity by 6FT. At 300 mM NaCl, inhibition was competitive (line). At low Na⁺ concentrations, and activation and inhibition are observed. B. The effect of 6FT on the activation of α-thrombin by Na⁺. Conditions were identical to those in Figure 4.2.
Figure 4.3.
Figure 4.4 Kinetics of inhibition of slow α-thrombin by 6-fluorotryptamine. A. Progress curves of chromogenic activity in increasing substrate. B. Double reciprocal plot of activity and substrate. Buffer was 10 mM Tris, 0.15 M choline chloride, 0.5 mg/mL PEG6000, pH 8.1. Chromogenic TH substrate was 200 μM.
Figure 4.4.
TABLE 4.1

Influence of 6FT on $V_{\text{max}}$ and $K_m$ of chromogenic TH.

<table>
<thead>
<tr>
<th>6FT</th>
<th>$V_{\text{max}}$ (mOD/min)</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>10.7</td>
<td>9.10</td>
</tr>
<tr>
<td>2 mM</td>
<td>11.6</td>
<td>10.1</td>
</tr>
<tr>
<td>4 mM</td>
<td>14.5</td>
<td>16.2</td>
</tr>
<tr>
<td>6 mM</td>
<td>18.7</td>
<td>25.1</td>
</tr>
<tr>
<td>8 mM</td>
<td>17.2</td>
<td>23.1</td>
</tr>
<tr>
<td>10 mM</td>
<td>17.1</td>
<td>25.4</td>
</tr>
</tbody>
</table>
Figure 4.5 Kinetics of mzIIa-F1 inhibited by 6-fluorotryptamine (6FT). A. The effect of Na⁺ on the inhibition of mzIIa-F1 by 6FT. B. The influence of 6FT on the inhibition and activation of mzIIa-F1 by Na⁺. Conditions were identical to those in Figure 4.2.
Figure 4.5.
with the $\alpha$-thrombin portion of the molecule, since $\alpha$-thrombin lacks this moiety. The impact of Na$^+$ on F2 intrinsic fluorescence would prove to be an interesting experiment. It should also be emphasized that this study was done using mzIIa-F1 which most likely represents an intermediate form between mzIIa and $\alpha$-thrombin, with traits of both. So it may be the case that mzIIa only possesses an inhibitory Na$^+$ site and does not exhibit the Na$^+$ activation as does mzIIa-F1 and $\alpha$-thrombin.

Inhibition by proflavin is also a useful tool for probing apolar binding. Inhibition of $\alpha$-thrombin under slow and fast conditions is shown in Figure 4.6. Note that, similar to tryptamine and 6FT, inhibition is simple competitive at high Na$^+$ ($K_i = 31.5$ $\mu$M), but virtually no inhibition is observed in the absence of Na$^+$. Thus, proflavin exhibits properties of a simple competitive inhibitor which binds to slow $\alpha$-thrombin weakly, if at all. In order to test the hypothesis that the binding of 6FT facilitates the conversion to fast $\alpha$-thrombin, $\alpha$-thrombin in the presence of 10 mM 6FT was inhibited with proflavin (curve 3, Figure 4.6). If this hypothesis were true, proflavin would exhibit simple competitive inhibition (as does fast $\alpha$-thrombin). This is clearly not the case. In fact, the lack of inhibition is quite reminiscent of slow $\alpha$-thrombin. Therefore, 6FT must activate $\alpha$-thrombin by some other mechanism other than the slow $\rightarrow$ fast transition.

**4.3.2 ESR spin label studies of apolar ligands binding to the slow form of $\alpha$-thrombin.**

Since all previous studies of apolar binding to spin labeled $\alpha$-thrombin have been performed in high NaCl concentrations (generally 0.75 M NaCl), the effects of the binding of indole derivatives on the ESR spectra of spin labeled human $\alpha$-thrombin under slow conditions were investigated. The X-band ESR spectra of $m$-IV, $m$-V, and $m$-VII labeled
\( \alpha \)-thrombin are given in Figures 4.7 - 4.9. As stated earlier the ESR spectra of slow \( \alpha \)-thrombins are generally more immobilized than the corresponding Na\(^+ \) bound form. In the presence of tryptamine, 6FT, proflavin, or indole (at 20 mM, 20 mM, 400 \( \mu \)M, and 10 mM, respectively) the mobilities of the meta labels changed very little compared to the fast form (most 2T\( \lambda \)'s did not differ by > 1 G from the slow form). The only ligand found to dramatically affect label mobilities was Na\(^+ \), as previously described in Chapter III. A summary of these data, as well as those of the para labels is presented in Table 4.2. The addition of 0.3 M NaCl (both by addition from a 1.2 M NaCl stock and by dialysis vs. 10 mM Tris, 0.3 M NaCl, pH 6.5) resulted in ESR spectra comparable to those measured in 0.75 M NaCl, indicating that the effects on ESR spin labels are reversible. However, smaller more subtle differences were observed for proflavin binding to \( m \)-V \( \alpha \)-thrombin and indole to \( m \)-VII \( \alpha \)-thrombin. In the case of proflavin, a 3 G increase in 2T\( \lambda \) was observed upon the addition of proflavin. This is somewhat surprising because proflavin was previously found to not bind to slow \( \alpha \)-thrombin (sections 3.3.2 and 4.3.1). However, the concentration of proflavin is 4-fold higher than in Figure 4.6 and the presence of a spin label may induce \( \alpha \)-thrombin toward a more "fast-like" conformation since the label mimics a substrate. If this is the case, it is not surprising that proflavin binding would immobilize \( m \)-V, since both were found by X-ray studies to bind the S1 pocket (Boxrud et al., 1996). The mobilization of the \( m \)-VII labeled \( \alpha \)-thrombin in indole is somewhat interesting. This label has been shown to be sensitive to indole in the bovine species, but not human (Nienaber and Berliner, 1991). Thus, it is probable that the label is forced into a different location by the local conformation of slow \( \alpha \)-thrombin, which is changed by the binding of indole. This is supported by the
Figure 4.6 Inhibition of α-thrombin by proflavin in a) 0.15 M choline chloride b) 0.15 M NaCl and c) 10 mM 6-fluorotryptamine. The buffer used was 10 mM Tris, 0.5 mg/mL PEG 6000 pH 8.1.
Figure 4.7 The influence of apolar ligands on the X-band ESR spectra of slow α-thrombin labeled with m-IV spin label. Buffer is 10 mM Tris, 0.3 M choline chloride, pH 6.5.

a) m-IV α-thrombin in buffer  
b) m-IV α-thrombin in 200 μM proflavin  
c) m-IV α-thrombin in 150 μM receptor peptide
Figure 4.7.
Figure 4.8 X-band ESR spectra of m-V labeled slow α-thrombin in the presence of apolar ligands. Buffer was identical to that in Figure 4.7.

a) m-V α-thrombin in buffer
b) m-V α-thrombin in 200 μM proflavin
c) m-V α-thrombin in 20 mM tryptamine
d) m-V α-thrombin in 20 mM indole
e) m-V α-thrombin in 150 μM receptor peptide
Figure 4.8.
Figure 4.9 X-band ESR spectra of \( m \)-VII labeled slow \( \alpha \)-thrombin in the presence of apolar ligands. Buffer is identical to that in Figure 4.7.

a) \( m \)-VII \( \alpha \)-thrombin in buffer
b) \( m \)-VII \( \alpha \)-thrombin in 200 \( \mu \)M proflavin
c) \( m \)-VII \( \alpha \)-thrombin in 20 mM tryptamine
d) \( m \)-VII \( \alpha \)-thrombin in 20 mM indole
e) \( m \)-VII \( \alpha \)-thrombin in 150 \( \mu \)M receptor peptide
Figure 4.9.
**Figure 4.10** X-band ESR spectra of p-I labeled slow α-thrombin in various apolar ligands. The buffer was identical to that in Figure 4.7.

a) p-I α-thrombin in buffer  
b) p-I α-thrombin in 20 mM indole  
c) p-I α-thrombin in 20 mM 6FT  
d) p-I α-thrombin in 150 μM receptor peptide
Figure 4.11 X-band ESR spectra of p-III labeled slow α-thrombin with apolar ligands. The buffer was identical to that in Figure 4.7.

a) p-III α-thrombin in buffer
b) p-III α-thrombin in 20 mM indole
c) p-III α-thrombin after consecutive scans in 20 mM indole
d) p-III α-thrombin in 150 μM receptor peptide
Figure 4.11.
Figure 4.12 X-band ESR spectra of p-V labeled slow α-thrombin in the presence of apolar ligands. The buffer was identical to that in Figure 4.7.

a) p-V α-thrombin in buffer
b) p-V α-thrombin in 200 µM proflavin
c) p-V α-thrombin in 20 mM tryptamine
d) p-V α-thrombin in 20 mM indole
e) p-V α-thrombin in 150 µM receptor peptide
Figure 4.12.
A summary of $2T_1$ values obtained from spin labeled α-thrombin with several apolar ligand under slow α-thrombin conditions. The buffer is identical to that described in Figure 4.7.

<table>
<thead>
<tr>
<th>Label</th>
<th>choline</th>
<th>NaCl</th>
<th>Indole</th>
<th>proflavin</th>
<th>tryptamine</th>
<th>6FT</th>
<th>pep</th>
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<tr>
<td>m-IV</td>
<td>58.4</td>
<td>&lt;45</td>
<td>-</td>
<td>58.7</td>
<td>-</td>
<td>-</td>
<td>61.2 G</td>
</tr>
<tr>
<td>m-V</td>
<td>60.8</td>
<td>61.0</td>
<td>61.4</td>
<td>64.1</td>
<td>61.2</td>
<td>-</td>
<td>62.0</td>
</tr>
<tr>
<td>m-VII</td>
<td>62.3</td>
<td>47.5</td>
<td>60.6</td>
<td>62.0</td>
<td>-</td>
<td>61.0</td>
<td>63.5</td>
</tr>
<tr>
<td>p-I</td>
<td>62.4</td>
<td>59.7</td>
<td>66.0</td>
<td>-</td>
<td>-</td>
<td>60.6</td>
<td>61.4</td>
</tr>
<tr>
<td>p-III</td>
<td>&lt;45</td>
<td>&lt;45</td>
<td>~62</td>
<td>&lt;45</td>
<td>&lt;45</td>
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<td>p-V</td>
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<td>&lt;45</td>
<td>63.6</td>
<td>63.3</td>
<td>61.2</td>
<td>-</td>
<td>66.5</td>
</tr>
</tbody>
</table>
large change in 2T₁ which occurs upon Na⁺ binding.

Rpep (Thrombin receptor peptide; Acyl-Tyr-Glu-Pro-Phe-Trp-Glu-Asp-Glu-Glu) is a highly negatively charged peptide which is identical to the sequence of the portion of the α-thrombin receptor which binds anion exosite I (Coughlin, 1993). The binding of thrombomodulin (TM) or hirudin C-terminal peptide to α-thrombin's anion exosite I has been shown by Di Cera's laboratory to facilitate the conversion of slow to fast α-thrombin (Di Cera et al., 1996). From the spin label studies, it appears that this is not the case. All of the meta labels exhibited an increase in mobility upon the addition of rpep. Rpep binds anion exosite I, so this mobility change is most likely not due to a direct interaction (Vu et al., 1991). It is most likely a conformational change at the active site induced by rpep binding which is responsible for the change in label mobility. The possibility also exists that the rpep induces the formation of an α-thrombin-rpep-thrombin complex. Some evidence exists for this type of complex formation with hirudin C-terminal peptide, which is homologous to rpep (Rowand, 1990; Vitali et al., 1992; Priestle et al, 1993; Mathews et al., 1994; Chang et al., 1995) More likely is that the slow → fast conversion initiated by the binding to anion exosite I does not have exert the same effects on the spin labels. In other words, the rpep bound state is fast-like, but not identical to the Na⁺ bound form.

Results from the para labels (Figures 4.10 - 4.12) were somewhat different to those found above for meta labels. In the case of p-I (Figure 4.10), indole and Na⁺ exhibited spectral changes. Again the Na⁺ bound form is more mobile, although not as dramatically as seen with the meta labels (~3 G). It is not surprising that indole affects meta and para labels differently as this is the case in the fast form as well, although changes are more dramatic in the case of slow α-thrombin. The effect is even more evident in p-III α-
thrombin (Figure 4.11), where the addition of indole effects an enormous increase of over 15 G. In this case, both slow and fast $\alpha$-thrombin $2T_1$ values are less than 45 G. Also evident is a dramatic increase in the deacylation rate of the label. In spectrum c) this is illustrated by an overlay of spectra once a minute, clearly demonstrating this. The above data suggest that indole may be in direct contact with the spin label causing the drastic immobilization and deacylation. Increased deacylation rates may also be attributed to activation of the enzyme, although the deacylation rate was clearly activated more than the 2-3 fold enhancement seen with indole activation of esterase activity (Conery and Berliner, 1983).

4.3.3 Fluorescence studies of apolar ligand binding using bis-ANS.

The fluorescent probe bis-ANS (Figure 2.16) is useful in the study of apolar aromatic ligands for several reasons. One is that it is itself a hydrophobic probe and can give information about the apolar binding sites of $\alpha$-thrombin (see Chapter III). Secondly, the excitation and emission wavelengths (390 and 490 nm, respectively) are distant enough from the indole wavelengths of 280 and 350 nm. Because the concentrations of indole derivative is quite high, interference of the ligand becomes a serious problem. A third advantage of bis-ANS is that it can bind in a non-covalent or covalent complex. Upon irradiation with UV light, bis-ANS forms a covalent complex with the chaperon protein GroEL by an unknown mechanism probably involving radical formation (Seale et al., 1995).

In order to better characterize the $\alpha$-thrombin bis-ANS complex, the GroEL procedure was modified slightly and $\alpha$-thrombin bis-ANS complex was irradiated with UV light from a hand held lamp. Resulting polyacrylamide electrophoresis gels showed the
complex formation as fluorescent bands co-migrating with α-thrombin (data not shown). The fluorescence spectra of the covalently bound complex compared to the non-covalent complex are shown in Figure 4.13. The emission maximum for the covalent complex compared to the non-cavalent one was found to be blue-shifted by 26 nm (493 nm and 466 nm, respectively). The stoichiometry of binding was determined to be 2.8:1 by assuming the bound bis-ANS to have an extinction coefficient of 16,790 M\(^{-1}\) cm\(^{-1}\) at 385 nm, equal to that of the free label (Musci et al., 1985). This compares unfavorably to 1.0 for the non-covalent bis-ANS complex (Musci et al., 1985). One of the possible reasons for this is that the concentration of bis-ANS during labeling was considerable higher than reported by Musci et al. (1985), where concentrations were limited by the inner-filter effect and by available protein. However, when bis-ANS is titrated to higher concentrations, an apparent second site is present in fast α-thrombin. Therefore, it is entirely possible that other binding sites exist that were not found by Musci et al. (1985).

The fluorescence spectra of covalently labeled bis-ANS α-thrombin in 0.15 M choline chloride and 0.15 M NaCl are shown in Figure 4.14A. Note the large blue shift upon the binding of sodium, which is much more pronounced than that of the non-cavalent complex, indicating that the surface of fast α-thrombin is more hydrophobic than the corresponding slow form. A titration of the slow form with NaCl is shown in Figure 4.14B and a \(K_D\) of 6.0 mM was obtained. As was the case with the non-covalent bis-ANS complex, the presence of this fluorophore appears to enhance the Na\(^+\) affinity of α-thrombin.
Figure 4.13 Fluorescence spectra of bis-ANS α-thrombin which is bound a) non-covalently or b) covalently. The buffer is 10 mM Tris, 0.15 M NaCl, pH 7.0, 20 ± 1 °C. Both α-thrombin concentrations are 0.5 μM and the noncovalent bis-ANS total concentration is 10 μM.
Figure 4.13.
The effect of several ligands of \( \alpha \)-thrombin added to covalently labeled bis-ANS \( \alpha \)-thrombin is demonstrated in Figure 4.15. Surprisingly, the presence of hirudin did not alter the fluorescence spectra of covalent complex, since benzamidine is known to effect a bis-ANS displacement in the non-covalent complex (Musci \textit{et al.}, 1985). Since hirudin blankets the entire active site region (Vitali \textit{et al.}, 1992), one would expect a perturbation of the bis-ANS fluorescence, unless benzamidine binds in a site other than the active site. Another possible explanation is that bis-ANS prevents hirudin from binding, presumably by steric hinderance of the competing bis-ANS molecule. Although this is doubtful since the \( K_D \) for hirudin is in the picomolar range (Stone and Hofsteenge, 1986). Also interesting is the fact that TMD1(CS-) and TMD1(CS+) evoke a fluorescence change while hirudin does not. Since EGF domains 5-6 are known to interact with \( \alpha \)-thrombin in anion exosite I (the same site as the C-terminal peptide of hirudin), it is likely that this bis-ANS interaction corresponds to the binding of the fourth EGF domain of TM (Esmon, 1993). This domain has been implicated in the conversion of \( \alpha \)-thrombin from a coagulant to an anticoagulant (Ye \textit{et al.}, 1992). This would imply that the bis-ANS site overlaps the EGF 4 binding site. The effect of ATP on covalent bis-ANS \( \alpha \)-thrombin is consistent with non-covalent results reported by Musci \textit{et al.} (1985). They report a 5-fold enhancement of bis-ANS fluorescence in the presence of 2.0 mM ATP. The results in Figure 4.15 also show a 5-fold enhancement in the presence of 10.0 mM ATP. It is clear that the bis-ANS binding site is dramatically affected by the presence of ATP, either directly (binding sites overlap) or indirectly (conformation change of the protein), in both the covalently and non-covalently bound form.
As discussed earlier with Na\(^+\) binding to bis-ANS \(\alpha\)-thrombin, it appears that bis-ANS has enhanced ligand binding. The binding constants calculated for three ligands serotonin (data not shown), tryptamine (data not shown), and 6FT (Figure 4.16) to non-covalently bound bis-ANS \(\alpha\)-thrombin were 26.5, 16.1, and 6.4 \(\mu\)M under fast conditions. Again, it is not surprising that this would occur, since tryptamine and 6FT are positively charged and bis-ANS contains negatively charged moieties and may enhance the binding of these ligands. Also, hydrophobic interactions may play a role. Musci \textit{et al.} (1985) refer to an enhancement in bis-ANS fluorescence upon addition of benzamidine to free bis-ANS (no benzamidine). In fact, a similar enhancement was noted for 6FT (data not shown). However, in both cases the enhancement observed in the presence of \(\alpha\)-thrombin was significantly larger than that of free label, so the enhancement was caused by bound bis-ANS. In the case of 6FT, this enhancement was of the same order of the free label. In other words, in the presence and absence of \(\alpha\)-thrombin, a 25\% increase in intensity is noted, but the bound intensity is 5-fold higher for bound. These results are consistent with bis-ANS binding directly to 6FT (Figure 4.16) and tryptamine (data not shown). The fluorescence is enhanced when bis-ANS is bound to \(\alpha\)-thrombin, but the enhancement due to 6FT still is present. The differences between slow and fast \(\alpha\)-thrombin are also intriguing. As shown in Figure 4.16A, the dissociation constant for 6FT to bis-ANS slow \(\alpha\)-thrombin is greater than 10-fold weaker than fast (90.5 \(\mu\)M). This difference may due to the direct interaction of Na\(^+\) ions with bis-ANS or a conformational difference between slow and fast \(\alpha\)-thrombin. It is difficult to differentiate between the two in this experiment. One possibility for the location of apolar binding site is a hydrophobic patch just above anion exosite I (Figure
Figure 4.14 Fluorescence of bis-ANS slow vs. fast α-thrombin. A. Comparison of the fluorescence spectra of covalent bis-ANS α-thrombin under a) slow and b) fast conditions. Buffer was 10 mM Tris, 0.15 choline (slow) or NaCl (fast), pH 7.0, 20 ± 1 °C. B. Titration of covalently labeled bis-ANS α-thrombin with NaCl under slow conditions. Experiment was such that the ionic strength of the solution remained at 0.15 M.
Figure 4.13 bis ans cov vs. non

Figure 4.14.
Figure 4.15 Addition of 30 mM ATP, 2.0 μM TMD1(CS-), 1.0 μM TMD1(CS+), or 10 μM hirudin to covalently labeled bis-ANS α-thrombin. Conditions were identical to Figure 14.
Figure 4.15.
Figure 4.16 Titration of noncovalent bis-ANS α-thrombin with 6FT under A. slow B. and fast conditions identical to those in Figure 4.14.
4.17). To test the possibility of this being the strong site which 6FT binds, the titration described in Figure 4.16 was performed in the presence of 5 μg / mL 12 mer (the last 12 residues of the C-terminal end of hirudin which bind to anion exosite I; Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Asn) in an effort to compete with the 6FT binding. The results of this are shown in Figure 4.18. The presence of 12 mer appears to increase the change in fluorescence and actually enhances the binding of 6FT slightly (6.3 and 4.3 μM for the absence and presence, respectively). This is indicative of simultaneous binding of 6FT, bis-ANS, and 12 mer. However, since 12 mer does influence the fluorescence change, the peptide may bind near the 6FT site. From the crystal structure of hirudin bound to α-thrombin, this hydrophobic site is removed from the bound peptide by approximately 5-8 Å (Brookhaven file 2hpq.pdb; Bode et al., 1989). Thus, it is possible for the two to bind concurrently. This hydrophobic site also has important implications in the binding of TM and it is possible that small molecules such as tryptamine, 6FT, and serotonin play a role in this interaction as well (Mathews et al., 1994).

4.4 DISCUSSION

4.4.1 Kinetic studies of tryptamine and 6-fluorotryptamine.

It is apparent from this data that 6FT binds α-thrombin in an activation site and an inhibitory site (with K_i's of and 1.5 mM, respectively). The inhibitory site is almost certainly that found to be S1 found by crystallography. However, the origin of the activation site is unclear. There are several plausible candidates for this activation site including the apolar site described by Bode et al. (1992) consisting of the S2 and S3 subsites, a hydrophobic patch
Figure 4.17 The surface of the \( \alpha \)-thrombin crystal structure as solved by Bode et al. (1989). The black regions are patches of aromatic residues (Trp, Tyr, and Phe), while hydrophobic residues (Ala, Ile, Leu, Val, and Pro) are designated by dark grey. Note that three major hydrophobic patches are visible, the active site apolar groove (labeled A), anion exosite I (B), and the anion exosite II (C).
Figure 4.18 Titration of non-covalent bis-ANS with 6FT in the presence of 5.0 μg/mL 12 mer under fast conditions (Figure 4.13).
near exosite I (fibrinogen recognition site), a hydrophobic patch near exosite II (heparin site), and the solvent channel adjacent to the Na⁺ binding site. There exists indirect evidence for and against each of these four interesting sites. First, ESR spin labeling studies by Berliner and Shen (1977b) have shown the indole binding site to be near the catalytic site. This is the reason that Bode et al. (1992) assigned the S2 and S3 subsites to be this indole site. However, occupation of this site by indole would most likely result in inhibition if fibrinogen clotting since S2 and S3 are necessary for fibrinogen binding. This is not the case. In fact, activation of clotting is seen in the presence of indole. This does not rule out binding to the hydrophobic rim of the active site canyon, however, leaving fibrinogen enough space to nestle in the binding groove. In the case of the anion exosite I, the hydrophobic patch just above the active site canyon in the anion exosite is an important site in the interaction of TM with α-thrombin (Mathews et al., 1994) and it is likely that binding to this site has conformational implications on the active site, since the binding of TM has been shown to induce active site changes (Musci et al., 1988; Ye et al., 1992). Also, the presence of 12 mer (binds in the exosite) did enhance the fluorescence of bis-ANS in the presence of 6FT (Figure 4.18).

The most compelling evidence is probably for the hydrophobic patch in the vicinity of anion exosite II. The site is about 10 Å from the active site and could certainly evoke a conformational change in the active site. This site is presumably blocked in mzIIa-F1 due to the presence of F2 in that vicinity. This would explain the lack of activation of esterase activity observed for mzIIa-F1 (Figure 2.15). There is also precedence for activation by this site in the case of non-covalently bound F2. α-Thrombin exhibits a 3-fold enhancement of
esterase activity in the presence of F2 (Myrmeal et al., 1976). As was with indole no amidolytic activation has been observed. An intriguing possibility also arose from the crystal structure of 6FT bound to α-thrombin. A region of electron density (later fit to water molecules) was found to fit a series of three 6FT molecules in the solvent channel adjacent to the Na⁺ binding site. This binding would explain the activation of α-thrombin by binding to the Na⁺ site and inducing the slow → fast transition. However, results in this work indicate that 6FT does not induce the slow → fast transition (Figure 4.6). One also cannot rule out the possibility that a new site forms on slow form, since all of the crystal structures published thus far have been of the fast form of α-thrombin.

An interesting hypothesis providing an explanation of why TAME esterase activity of α-thrombin was activated by indole and α-thrombin amidolytic activity was not was put forward by Conery and Berliner (1983). This stated that since the rate limiting step in TAME cleavage by α-thrombin is the deacylation step and the acylation step in amidolytic substrates, the acylation complex is activated by indole, effecting more rapid deacylation. This would have the effect of increasing the rate of catalysis for those substrates limited by the deacylation step and not affect those which are not. The activation of slow α-thrombin can thus be achieved because α-thrombin has been found to limited by deacylation under slow conditions (Wells and Di Cera, 1992). Thus, under slow conditions α-thrombin amidolytic activity is activated by indole and indole derivatives.

The physiological effects on α-thrombin of platelet secretion of indole derivatives are still somewhat unclear. However, evidence appears to point toward these molecules being inhibitors of α-thrombin. When both slow and fast α-thrombin are present (as they
are under physiological conditions) secretion of high concentrations would serve to inhibit fast \( \alpha \)-thrombin, thus inhibit the formation further conversion of fibrinogen to fibrin. On the other hand, slow \( \alpha \)-thrombin would be inhibited to a much lesser degree (or even activated). Thus, the anticoagulation pathway would remain functional, resulting in removal of more \( \alpha \)-thrombin.
CHAPTER V

INTERACTIONS OF THE ACTIVE SITE OF t-PA WITH A DISTAL KRINGLE MOIETY

5.1 INTRODUCTION

5.1.1 Goals of the study.

Tissue plasminogen activator (t-PA) is unique among serine proteases in that its zymogen form, single chain t-PA (sct-PA) possesses a substantial amount of activity (17 - 33 % of that of the active form two-chain t-PA (tct-PA); Tate et al., 1987; Boose et al., 1989). It has been postulated by Nienaber et al. (1992) that the single chain form can exist in two conformations, active and inactive. Sct-PA resides generally in the inactive form. However, upon binding a substrate, adopts the active form. This is supported by fluorescence data, in which a 50% decrease in emission intensity of anthraniloyl-sct-PA was observed upon conversion from sct-PA to tct-PA with V8 protease (the structure of p-nitrophenyl anthranilate is presented in Figure 2.16; Nienaber et al., 1992). The sulfonyl fluoride spin labels described in Figure 2.1 mimic the substrate in that they form a tetrahedral complex analogous to the tetrahedral intermediate formed upon acylation of the amidolytic substrate. With these labels, as well as several fluorescent labels, Nienaber et al. (1992) little or no differences between the sct-PA and tct-PA. This is presumably because these labels
mimic the substrate and push the enzyme into the active conformation (similar to that of tct-PA), therefore no changes are observed upon activation. Only anthraniloyl set-PA exhibited significant changes upon tct-PA conversion or conformational changes induced by occupation of the lysine binding site of kringle 2 (Nienaber et al., 1992).

The spin label SL3, which is analogous to p-nitrophenyl anthranilate and p-nitrophenyl acetate is employed in this study to monitor conformational differences between set-PA and tct-PA. It was also found by Nienaber (1990) that anthraniloyl set-PA exhibited changes in fluorescence upon occupancy of its lysine binding site located on kringle 2. Because of this, the effect of lysine and several analogues (arginine and ε-amino caproic acid) was also examined. Thus, the goal of this study was to provide supporting or conflicting evidence to this two conformation hypothesis.

5.1.2 Rationale of the approach and summary of the results.

The spin label SL3 was chosen for studies of set-PA and tct-PA because the acylated protein exists in a trigonal complex with the spin label. Presumably, this label does not trigger conversion of set-PA to the active conformation because it does not mimic a substrate and bind to the oxyanion hole. The major obstacle in this study is the fact that the deacylation rate constant for SL3 is quite high at pH 7.0, subsequently most studies have been done at pH 3.0 and 5.0. Even at pH 5.0, however, the appearance of deacylated free label is quite apparent. Fortunately, these lines are quite distinguishable from the bound spectra.

The ESR spectra of SL3 labeled t-PA were found to be strongly immobilized ($2T_1 >$
64 G for all spectra) under all conditions examined in this work. A slight additional immobilization ($\Delta 2T_1 = 1.5$ G) was noted upon conversion from sct-PA to tct-PA, also evident was a substantial increase in the deacylation rate (evident by the appearance of free signal). Not surprisingly, an increase in deacylation was also noted upon an increase in pH from 3.0 to 7.0, although no change in $2T_1$ was noted. More interesting, was the increase in both deacylation and immobilization (1.5 G) upon addition of 20 mM $\varepsilon$-aminocaproic acid ($\varepsilon$-ACA). This ligand is known to increase amidolytic activity of both sct-PA and tct-PA (de Munk et al., 1989). The results here show that the presence of $\varepsilon$-ACA induces sct-PA into a conformation which resembles that (at least spectroscopically) of tct-PA. The effect of arginine is somewhat different. The presence of 0.17 M arginine stabilized both SL3 labeled sct-PA and tct-PA from deacylation and induced no change in the immobilization of the label.

5.2 MATERIALS AND METHODS

5.2.1 Materials.

$\varepsilon$-Amino caproic acid, arginine, and plasmin were obtained from Sigma Chemical Company. SL3 was synthesized previously according to Berliner and McConnell (1966). All other chemicals were of reagent grade or higher and used without further purification.

5.2.2 Proteins.

5.2.2.1 t-PA

Human recombinant sct-PA was a generous gift of Dr. W. Bennett (Genetech, San
Francisco, CA) as a lyophilized powder. Human plasmin was purchased from Sigma. Solutions of sct-PA were prepared by dissolving in 80 mM phosphate, 0.17 M arginine, 0.0008 % Tween 80, pH 7.0. Tct-PA was prepared by incubating sct-PA two hours at room temperature with 0.24 U/mL plasmin. Activation progress was monitored by electrophoresis.

5.2.3 ESR spectroscopy.

5.2.3.1 Spin labeling.

Spin labeling of tPA was accomplished by incubating tPA at a concentration of 50 μM with 5-fold excess of SL3 (Figure 5.1) in 80 mM phosphate, 0.17 M arginine, 0.0008 % Tween 80, pH 7.0 for 10 min. at room temperature. The labeled t-PA was separated from free label using Bio-Rad Bio-Spin P-6 columns equilibrated in the above buffer at pH 3.0. Use of these columns is described in section 2.2.4.1. ESR measurements were carried out immediately after removal of free spin label. Occasionally, exhaustive dialysis vs. phosphate buffer at pH 3.0 was employed to remove the free SL3.

5.2.3.2. ESR Spectra

ESR spectra were obtained as described in section 2.2.4.2. Deacylation experiments were performed by repeated scan over the same peak with the time and peak height recorded. The increase of free label (peak height) was then fit to an exponential increase.

5.2.4 Electrophoresis.

Electrophoresis is described in section 2.2.3.
5.3 RESULTS

5.3.1 Comparison of one-chain and two-chain t-PA.

The spin label SL3 (shown in Figure 5.1) was employed to study the active site of both sct-PA and tct-PA. Figure 5.1 shows a comparison of the two labeled proteins at pH 3.0 (spectra a and b) and at pH 5.0 (spectra c and d). In this case, sct-PA was labeled and cleaved with 0.24 U / mL of plasmin in the presence of 0.17 M arginine, verified by use of SDS-PAGE (data not shown). As Figure 5.1 reveals, both at pH 3.0 and pH 5.0, the nitroxide moiety of SL3 is highly immobilized in both sct-PA and tct-PA (see Table 5.1 for $2T_1$ values). There is very little apparent difference between the active site environments that the nitroxide of SL3 probes at both pH's. However, the appearance of the deacylation product, represented by three sharp lines (Figure 5.1, spectrum b) does suggest that the two chain form is more active. Thus, indirectly this provides evidence for the tct-PA conversion. The free line signal which appears in spectra c) and d) is a result of increased deacylation due to the increased pH. This result is somewhat surprising because SL3 is somewhat analogous to p-nitrophenyl anthranilate, in which differences in fluorescence emission between labeled sct-PA and tct-PA were previously found (Nienaber et al., 1992). It should also be noted that no differences in sulfonylfluoride spin label spectra could be discerned (Nienaber et al., 1992). It is possible, however, that the smaller proxyl nitroxide ring (vs. the anthraniloyl ring) is able to nestle into a local environment which is unchanged between tct-PA and sct-PA. Proxyl rings are also intrinsically more rigid and more likely to give rise to a highly immobilized spectrum. It is quite clear that the SL3 spin label probes a very restricted
Figure 5.1 X-band ESR spectra of SL3 labeled human sct-PA and tct-PA in 80 mM phosphate, 0.17 M arginine, 0.0008% Tween 80, pH 3.0 and 5.0.

a) SL3 labeled sct-PA at pH 3.0
b) SL3 labeled tct-PA at pH 3.0
c) SL3 labeled sct-PA at pH 5.0
d) SL3 labeled tct-PA at pH 5.0
Figure 5.1.
locus of the t-PA active site. This is supported by labeling studies with a six-membered pyrrolidine ring replacing the proxyl ring in which very poor labeling was observed (data not shown).

5.3.2 Effect of binding to the lysine binding site on the active site of t-PA.

As discussed in section 1.2.5, fibrin binding to sct-PA activates its chromogenic activity (Wilhelm et al., 1990; Nienaber, 1990). Interaction with soluble fibrin fragments has also been shown to affect active site labeled sct-PA (Nienaber, 1990). The "communication" between the active site and distant fibrin binding is facilitated by the lysine binding site of kringle 2 as well as interactions with kringle 1 and the finger domain of t-PA (Higgins and Bennett, 1990). Studies of t-PA activity in the presence of arginine, lysine and ε-ACA have shown that sct-PA, but not tct-PA, activity is enhanced in the presence of either of these ligands (Higgins and Vehar, 1987; de Munk et al., 1989; Higgins and Bennett, 1990). Figure 5.2 is a comparison of SL3 labeled sct-PA in the presence and absence of 0.17 M arginine at pH 3.0 (spectra a and b), respectively) and in the presence and absence of 10 mM ε-ACA at pH 5.0 (spectra c and d), respectively). Again, it is apparent that there is little difference between the two forms at either pH.

In order to assure that conclusions made about spectra taken at pH 3.0 correlate with the actual enzyme conformation at pH 7.0, the ESR spectra of SL3 labeled sct-PA was measured at pH 3.0, 5.0 and 7.0. While pH 7.4 would be ideal for t-PA studies because of its physiological relevance, the deacylation rate of SL3 at this pH precludes any extensive ESR study at this pH. Thus, more acidic conditions were used for most of this study. These
Figure 5.2 X-band ESR spectra of SL3 labeled sct-PA in the absence and presence of arginine and e-ACA. Buffer is 80 mM phosphate, 0.0008 % Tween 80, pH 3.0 or 5.0.

a) SL3 labeled sct-PA at pH 3.0
b) SL3 labeled sct-PA in 0.17 M arginine at pH 3.0
c) SL3 labeled sct-PA at pH 5.0
d) SL3 labeled sct-PA in 100 mM e-ACA at pH 5.0
Figure 5.2.
Figure 5.3  X-band ESR spectra of SL3 labeled sct-PA at pH a) 3.0, b) 5.0, and c) 7.0. Buffer is 80 mM phosphate, 0.0008 % Tween 80.
Figure 5.3.
spectra are shown in Figure 5.3. Note that once again little change in the mobility of the label has occurred, and 2T₁ values of 65, 64.5, and 65 G for pH's 3.0, 5.0, and 7.0, respectively. It is clear from these spectra that little or no change in label environment occurs in sct-PA over a pH range of 3.0 to 7.0. As expected, the observed deacylation rate increased dramatically with increasing pH. From this data, it appears that spectra taken at pH 3.0 can be utilized to make conclusions about t-PA at pH 7.0.

The activation of sct-PA by ε-ACA is thought to be representative of activation by fibrin. As with tct-PA vs. sct-PA labels with tetrahedral geometry at the active site serine show no effects of the binding of ε-ACA and the anthraniloyl sct-PA, a trigonal complex, exhibited changes upon ε-ACA binding (Nienaber et al., 1992). The occupation of the lysine binding site of kringle-2 is thought to facilitate the sct-PA to adopt its activated tct-PA-like conformation. Figure 5.4 shows the effect of ε-ACA binding on SL3 labeled sct-PA at pH 3.0. A slight immobilization (Δ2T₁ = 1.5 G) of the nitroxide and an increase in deacylation were observed, both occurring between 10.0 and 100.0 mM. This provides evidence for conformational changes occurring at the active site as a result of binding at the distal kringle-2 lysine binding site.

A comparison of the deacylation rate at pH 7.0 of SL3-sct-PA in the presence and absence of 0.17 M ε-ACA is shown in Figure 5.5. Half lives of 18.4 min and 22.09 min were obtained for absence and presence of 0.17 M ε-ACA, respectively. The increase in rate constant due to the presence of ε-ACA clearly suggests that ε-ACA converts sct-PA into a more active conformation, most probably resembling tct-PA. The effect of ε-ACA on the ESR spectra of SL3 labeled tct-PA is shown in Figure 5.6. In this case, there is no change
**Figure 5.4** X-band ESR spectra of SL3 labeled sct-PA in increasing ε-ACA. Buffer is 80 mM phosphate, 0.0008 % Tween 80, pH 3.0.

a) SL3 labeled sct-PA in no ε-ACA, pH 3.0  
b) SL3 labeled sct-PA in 2 mM ε-ACA, pH 3.0  
c) SL3 labeled sct-PA in 10 mM ε-ACA, pH 3.0  
d) SL3 labeled sct-PA in 100 mM ε-ACA, pH 3.0
Figure 5.4.
Figure 5.5 Deacylation of SL3 labeled sct-PA as monitored by free line signal. Buffer was 80 mM phosphate, 0.0008 % Tween 80, pH 7.0. A. In the absence of ε-ACA. B. In 20 mM ε-ACA.
Figure 5.6  X-band ESR spectra of SL3 labeled tct-PA in increasing $\epsilon$-ACA. Buffer is 80 mM phosphate, 0.0008 % Tween 80, pH 3.0.

a) SL3 labeled tct-PA in no $\epsilon$-ACA, pH 3.0
b) SL3 labeled tct-PA in 2 mM $\epsilon$-ACA, pH 3.0
c) SL3 labeled tct-PA in 10 mM $\epsilon$-ACA, pH 3.0
d) SL3 labeled tct-PA in 50 mM $\epsilon$-ACA, pH 3.0
Figure 5.6.
TABLE 5.2

Summary of the observed $2T_1$ values for SL3 t-PA studies

<table>
<thead>
<tr>
<th>chain</th>
<th>pH</th>
<th>Arg</th>
<th>e-ACA</th>
<th>$2T_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>64.0 G</td>
</tr>
<tr>
<td>sc</td>
<td>3.0</td>
<td>-</td>
<td>20 mM</td>
<td>66.5</td>
</tr>
<tr>
<td>sc</td>
<td>3.0</td>
<td>-</td>
<td>100 mM</td>
<td>*66.5</td>
</tr>
<tr>
<td>sc</td>
<td>3.0</td>
<td>0.17 M</td>
<td>-</td>
<td>65.0</td>
</tr>
<tr>
<td>sc</td>
<td>3.0</td>
<td>0.17 M</td>
<td>2.0 mM</td>
<td>65.0</td>
</tr>
<tr>
<td>sc</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>*64.5</td>
</tr>
<tr>
<td>sc</td>
<td>5.0</td>
<td>0.17 M</td>
<td>-</td>
<td>64.5</td>
</tr>
<tr>
<td>sc</td>
<td>7.0</td>
<td>0.17 M</td>
<td>-</td>
<td>*~65</td>
</tr>
<tr>
<td>tc</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>66.5</td>
</tr>
<tr>
<td>tc</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>tc</td>
<td>3.0</td>
<td>-</td>
<td>100 mM</td>
<td>66.5</td>
</tr>
</tbody>
</table>

* significant deacylation
in label mobility or deacylation rates. This is consistent with the hypothesis that ε-ACA acts to convert sct-PA to a more active conformation. In the case of tct-PA this conversion has already occurred, thus there is no apparent effect on the label.

5.4 DISCUSSION

The ESR spin labeling results of this work are complementary to those found by Nienaber et al. (1992) in that changes in spectral properties of sct-PA were noted upon conversion to tct-PA or the binding of ε-aminocaproic acid. Although the results here are less dramatic than the large quenching observed with anthraniloyl-t-PA, the results are no less compelling. It is clear that the active site of sct-PA becomes more like that of tct-PA upon the binding of ε-ACA. The fact that tct-PA does not exhibit any spectral changes in the presence of ε-ACA also serves as a negative control, suggesting that this result is not due to any nonspecific effects of the ε-ACA.

The immobility of this label on both forms of t-PA is not surprising. Berliner and McConnell (1966) noted that this label is quite immobilized on α-chymotrypsin, a much smaller protein than t-PA (24 kda vs 59 kda). Also of note is that an analogous label with a piperidinyl (six-membered ring), exhibits extremely poor labeling, and presumably cannot fit into the active site cleft. Thus, it appears that this label does not have a lot of room for rotation and is thus immobilized.

It is remarkable that the mobilities of the spin label on both sct-PA and tct-PA are so similar, considering that tct-PA is an active form and sct-PA is not. The possibility that these
two labels probe a region of the active site which is virtually identical in the two forms is intriguing. It would mean that this region of the enzyme active site is formed in the zymogen form. However, since little data for active site spin labeled zymogens exists, it is difficult to assess this possibility. The possibility also exists that because the immobilization is so great as to near the upper limit of $2T_1$ (about 72 G), the spectra is insensitive to changes in label motion. This possibility is certainly reasonable considering the highly immobilized spectra obtained (65 G). Also, small differences were observed in the $2T_1$ values between the two forms of t-PA.
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