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# 5-FLUORO-TRYPTOPHAN AS A PROBE FOR FLUORESCENCE AND FLUORINE 19 NMR STRUCTURE-FUNCTION STUDIES: ANALYSIS OF 5-FLUORO-TRYPTOPHAN SUBSTITUTED SOLUBLE TISSUE FACTOR

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1998

UMI Number: 9820597

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/29/98
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# Abstract

# 5-FLUORO-TRYPTOPHAN AS A PROBE FOR FLUORESCENCE AND FLUORINE 19 NMR STRUCTURE-FUNCTION STUDIES: ANALYSIS OF 5-FLUORO-TRYPTOPHAN SUBSTITUTED SOLUBLE TISSUE FACTOR

by

Jennifer Zemsky

Adviser: Professor J.B.A. Ross

Both protein fluorescence and $^{19}$F NMR spectroscopies are highly sensitive tools for probing the local environments of proteins. However, their usefulness becomes limited in complex multi-component systems because the components become indistinguishable. One approach to characterization of multi-protein or protein-DNA systems is to label one component such that it can be selectively observed. Proteins may be labeled either intrinsically or extrinsically. Intrinsic probes have two advantages over extrinsic probes. First, intrinsic probes are site specific. Extrinsic probes often label proteins randomly. Second, intrinsic probes often perturb protein structure less than extrinsic probes. Tryptophan analogs have proven to be useful intrinsic probes both for fluorescence and $^{19}$F NMR spectroscopies. Substitution on the indole ring of tryptophan can result in altered spectroscopic properties. In this study, the utility of 5-fluoro-tryptohan as an intrinsic probe has been characterized. 5-fluoro-tryptophan has two advantages as a probe. First, 5-fluoro-tryptophan may be used as both a $^{19}F$ NMR and fluorescence probe, allowing complementary data to be gathered on the same sample by both techniques. Second, 5-fluoro-tryptophan perturbs protein structure minimally. In this study, 5-fluoro-tryptophan was substituted for tryptophan in soluble human tissue factor and its single tryptophan replacement mutants. Both the $^{19}F$ NMR and fluorescence spectra of these proteins have been characterized. The data on the local environment has been characterized with respect to previous studies characterizing the tryptophan fluorescence of these proteins.

## Acknowledgments

First and foremost, I would like to thank my advisor J.B.A. "Sandy" Ross without whom it would not have been possible to complete my dissertation. He has been an incredible teacher both academically and technically.

My thanks also go to Elena Rusinova whose technical and conceptual knowledge has been invaluable in my transition to fluorescence spectroscopy.

Thanks to Dr. Linda A. Luck for all her patience and time during the NMR runs and for all the effort she took in helping to understand the data. Also, I would like to thank her for her hospitality during my trips up north for the NMR runs.

I would also like to note my appreciation to my committee, Dr. William R. Laws and Dr. Ronald A. Kohanski, for their guidance and for their roles as devil's advocate.

I would like to thank Arthur A. Guffanti and David B. Hicks for their all their technical help over the years.

Last, but not least, my sincerest thanks to my husband, family and friends whose support has been inestimable.

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# CHAPTER 1

# General Introduction and Literature Review

## General Introduction:

Both NMR and intrinsic protein fluorescence spectroscopy have been widely used to study protein structure and dynamics. Application of both methods can become limited, however, as signals become too complex to separate into components in either large proteins, multi-protein or protein-DNA systems. One approach to simplifying protein signals in both NMR and fluorescence spectroscopy is substitution of amino acid analogs for residues of interest, limiting the signal to discrete regions. In this study, the use of 5-fluoro-tryptophan (5F-Trp) has been analyzed using both a NMR and fluorescence spectroscopies. Specifically, 5F-Trp has been used to replace tryptophan (Trp) in a protein that was then characterized for fluorescence properties and analyzed via $^{19}$F NMR spectroscopy.

### Spectral Enhancement of Proteins

One approach to simplifying signals from both fluorescence and NMR spectroscopies is the selective use of probes, either extrinsic or intrinsic to the protein being studied. Replacement of Trp with analogs of Trp that have altered spectroscopic characteristics allows the placement of an intrinsic probe within a protein (Ross et al.. 1997). One advantage of using these analogs is that proteins containing analogs can be selectively observed in the presence of non-analog replaced proteins or DNA. Many analogs of Trp that have alterations on the indole ring have altered absorbance and fluorescence characteristics, resulting in shifted absorbance relative to Trp. Those analogs that are red-shifted relative to Trp can be selectively excited in a range in which Trp has essentially no absorption. The ability to selectively excite the analog allows observation of the fluorescence emission spectra of one protein component in the presence of a second component, either protein or DNA, without interference from the second. Similarly, for $^{19}$F NMR, labeling of just one component with a fluoro-Trp (F-Trp) analog renders the second component silent. The labeled component can be specifically monitored in the presence of other components, keeping the signals relatively simple.

Working with 7-azatryptophan (7A-Trp) and tryptazan, Schlesinger (1968) was the first to note the altered spectroscopic properties of the Trp analogs. While fluorinated amino acids analogs, primarily of the aromatic amino acids, have been incorporated into proteins for many years to be used in $^{19}$F NMR studies (Gerig, 1994; Danielson & Falke, 1996), the potential of Trp analogs for generating spectrally enhanced proteins for fluorescence studies had been overlooked until recently. Recently, many groups have incorporated Trp analogs into proteins expressed in bacteria with varying degrees of success (reviewed in Ross et al., 1997). While some proteins will not tolerate substitution of Trp by an analog, most proteins fold in a stable state and form active proteins, as based upon activity measurements (Ross et al., 1997; Gerig, 1994; Danielson & Falke, 1996). To further examine the structural effects of replacing Trp with an analog, Lau and Gerig (1997) performed dynamic simulations to determine the effect of substituting 6F-Trp for Trp in *Escherichia coli* (*E. coli*) dihydrofolate reductase. Based upon their results, they concluded that the substitution had minimal effects on either structure or dynamics. Similarly, Wong and Eftink (1997) studied the effects of 5-hydroxytryptophan (5OH-Trp) and 7A-Trp on the structure and stability of staphylococcal nuclease using circular dichroism and fluorescence anisotropy. They determined that based upon the circular dichroism measurements, the analog containing proteins had similar secondary structure to the wild type. The 7A-Trp substituted variant was found to be less stable than the wild type or 5OH-Trp substituted variant, and followed a different folding pathway than the other two proteins.

Shen et al. (1996) demonstrated that a protein in which Trp has been replaced with either 5OH- Trp or 7A-Trp can in fact be selectively excited in the presence of Trp containing proteins and be used to monitor changes in the local environments of the 7A-Trp containing protein. They replaced the Trp with either 5OH-Trp or 7A-Trp in the activation domain of the Herpes virus protein VP16. They then examined its interactions with either the TATA binding protein or transcription factor B. They were able to determine that VP16 interacts differently with the two partner proteins.

**Fluorinated Analogs of Tryptophan:**

Fluorinated analogs of tryptophan have primarily been used in $^{19}$F NMR spectroscopy experiments. Most commonly used in studies are the 4, 5 or 6F-Trp analogs. The fluorine atom on the indole ring provides a means to specifically probe the local environment of a single atom. Fluorine has several advantages as a NMR probe. The isotope is 100% abundant and has 83% the sensitivity of protons. As noted above, fluorine is minimally perturbing to protein environments. Additionally, the lone pair of electrons on the fluorine atom control the chemical shift and are affected by both the van der Waals and electrostatic interactions of neighboring residues, making the $^{19}$F chemical shift highly sensitive to local environment (reviewed in Gerig, 1994; Danielson & Falke, 1996). By contrast, fluorinated Trp has been used in relatively few fluorescence studies and little is known about the absorption or emission characteristics of the analogs. 4F-Trp is known to be non-fluorescent. First noted by Bronskill and Wong (1988), who proposed that replacement of Trp by 4F-Trp could be used to create proteins to act as non-fluorescent partners in complexes, allowing monitoring of the fluorescent component. Hott and Borkman (1989) later found that 4F-Trp is prone to photolysis and forms fluorescent products limiting the utility of 4F-Trp. However, since 4F-Trp has blue-shifted absorption, excitation of partner proteins at wavelengths of 300 nm or longer minimizes the decomposition of the 4F-Trp residues (Ross et al., 1997). In the same study that they characterized 4F-Trp, Bronskill and Wong also characterized the absorption and fluorescence emission spectra of 5 and 6F-Trp. The 6F-Trp absorption spectrum is entirely overlapped by the Trp absorption spectrum, making it impossible to selectively excite in the presence of Trp. 5F-Trp, in contrast, has a red-shifted absorbance spectrum relative to Trp. The fluorescence emission spectrum of 5F-Trp is highly similar to that of Trp, being only slightly red-shifted relative to Trp while the emission spectrum of 6F-Trp is more significantly red-shifted.

Robertson et al. (1977) correlated $^{19}F$ NMR and fluorescence data in a study in which they characterized the histidine-binding protein of *Salmonella typhimurium*. They replaced the Trp residues with 5F-Trp. $^{1}H$ NMR, $^{19}F$ NMR and fluorescence emission measurements were made for the protein in the absence and presence of ligand, partial ligands and non-ligands. They found that the $^{19}F$ NMR data and the fluorescence emission spectra indicated that one or more Trp residues become less solvent exposed upon binding ligand, indicating that $^{19}F$ NMR and fluorescence emission spectra yield supporting data and are complementary methods.

**Tryptophan Analog Incorporation:**

Biosynthetic incorporation of tryptophan analogs was first examined in studies characterizing bacterial growth and protein synthesis. Pardee et al. (1956), studied the incorporation of 7A-Trp into the proteins of *Escherichia coli* (*E. coli*) and bacteriophage $T_2$. They found that 7A-Trp could support growth of an *E. coli* Trp auxotroph and that they could demonstrate incorporation of 7A-Trp into proteins. Lark (1969) was able to use 5-methyltryptophan (5M-Trp) to support growth of another *E. coli* Trp auxotroph, again finding that the Trp analog could be used to replace Trp in proteins. Barlati and Ciferri (1970) had similar results in a Trp auxotroph of *Bacillus subtilis* (*B. subtilis*) using 5M-Trp and 5OH-Trp. Later, Pratt and Ho (1975) found that the 4, 5 or 6F-Trp analogs could support growth of an *E. coli* Trp auxotroph and be successfully incorporated into proteins.

While all of the analogs used in these studies will support growth of the *E. coli* and *B. subtilis* Trp auxotrophs, all of the groups found that the analogs had an associated toxicity relative to growth on Trp. This toxicity suggests that either the protein synthesis machinery does not recognize the analogs adequately or that incorporation of the analogs into essential proteins results in malformed proteins. Studies of the substrate preferences of tryptophanyl-tRNA synthetase have since demonstrated that successful incorporation is in part dependent upon the choice of analog, with some analogs recognized better by the enzyme than others (Xu et al., 1989; Hogue & Szabo, 1993). The advent of recombinant DNA technology allowed for any protein of interest to be expressed under a regulated promoter so that synthesis of a protein can be limited to discrete phases of cell growth. Expression of proteins that are targets for Trp analog replacement under tightly controlled promoters in Trp auxotrophs has avoided the toxicity noted in the earlier studies (Ross et al., 1997; Hogue, 1994). Having proteins under tightly regulated promoters allows growth of cell cultures to high density on Trp containing medium, followed by a switch to minimal medium supplemented with the Trp analog of interest. Protein synthesis is induced only after the media is switched. Using this approach, many proteins have been expressed with levels of analog incorporation nearing 95% (Ross et al., 1997). However, other proteins will not tolerate substitution of the analogs yielding poorly incorporated and/or insoluble proteins (Ross et al., 1997; Hasselbacher et al., 1995), suggesting that the toxicity associated with the analogs may be the result of essential proteins not folding properly.

## Tissue Factor:

Tissue Factor (TF) initiates blood clotting in both the intrinsic and extrinsic pathways. It performs this role under conditions of both homeostasis and thrombosis. Unlike other coagulation factors, TF requires no further modification for activity. Activation instead is regulated by physical separation. Other than TF, coagulation factors circulate freely in the blood stream. By contrast, TF is kept separate from the blood stream. TF, under normal conditions, localizes in tissues that are at boundaries between the body and the environment and between organs. Specifically, TF is expressed in the adventitia of blood vessels, in the nervous system, the brain, the lung, and the placenta and around the liver, spleen and kidney. By contrast, TF expression is found to be excluded from vascular tissue (Semeraro & Colucci, 1997; Østerud, 1997; Nemerson, 1995; Edgington et al., 1997).

Improper expression of TF is associated with several pathological conditions. Immune response can trigger expression of TF on monocytes and endothelial tissue in the vasculature. Exposure to endotoxin or gram-negative bacteria results in expression of TF in the blood stream leading to lethality. The rate of lethality has been correlated with the level of TF expression (Drake et al., 1993; reviewed in Semeraro & Colucci, 1997; Ruf & Edgington, 1994). Treatment of baboons exposed to *E. coli* with tissue factor pathway inhibitor (TFPI) reduces the mortality rate associated with the increased expression of TF, further supporting the role of TF in lethality (Semeraro & Colucci, 1997; Ruf & Edgington, 1994). Abnormal expression of TF is also associated with both atherosclerosis and tumor metastasis. Expression of TF is induced by an unknown mechanism in macrophages, smooth muscle cells and monocytes associated with athersclerotic plaques. The presence of TF in these plaques is associated with lethal thrombus generation (Wilcox et al., 1989; Taubman et al., 1997; Ruf & Edgington, 1994). Treatment with lipoprotein-associated coagulation inhibitor (also known as TFPI) has been shown to inhibit thrombotic reocclusion of arteries after vascular injury (Haskel et al., 1991) supporting the role of TF in lethal thrombus formation. TF expression is also found to be necessary for the development of a number of cancers. The presence of

TF in leukemias and in solid tumors of lung, pancreas, breast and colon is associated with disseminated intravascular coagulation (Haskel et al., 1991). It is also believed that expression of tissue factor is essential for metastasis of certain cancers (Mueller et al., 1992).

In addition to its role as regulator of coagulation, TF is suspected to have a role in signal transduction systems. Binding of TF by Factor VIIa (VIIa) has been found to transiently induce $Ca^{2+}$ oscillations in multiple cell lines (Röttingen et al., 1995; Østerud, 1997). Additionally, the cytoplasmic tail of TF has been found to be phosphorylated in a Protein Kinase C dependent manner (Zioncheck et al., 1992), further suggesting a role mediating signal transduction in response to tissue damage.

TF is transmembrane receptor type protein, structurally homologous with the cytokine receptor family (Østerud, 1997; Muller et al., 1996; Edgington et al., 1997). VIIa initiates coagulation as a result of the binding of TF in the blood stream. TF is exposed to the blood stream as a result of tissue damage. Formation of a TF-VIIa complex results in activation of factors X and IX, resulting in stimulation of both the intrinsic and extrinsic coagulation pathways. Activated factor X then maintains stimulation of coagulation while the TF-VIIa is rapidly inhibited by TFPI thereby limiting activation of coagulation (Østerud, 1997; Nemerson, 1988; Edgington et al., 1991). Activation of the complex is dependent upon the presence of $Ca^{2+}$ ions and lipids (Paborsky et al., 1991; Fiore et al., 1994; Bach & Rifkin, 1990; reviewed in Nemerson, 1995). Furthermore, it has been observed that the lipid composition has an effect on the activity of the complex, with acidic phospholipids stimulating TF-VIIa activity (Neuenschwander & Morrissey, 1995; Bach et al., 1986).

The structures of TF and VIIa both separately and in complex have been studied by multiple approaches. Many structural studies have used a soluble version of TF (sTF) in which the transmembrane and cytoplasmic domains have been eliminated. sTF can be expressed at higher levels and made crystallization possible by elimination of the lipid domains. High-resolution crystal structures of sTF alone and in complex with VIIa have been analyzed. The sTF crystal structure, most recently refined to 1.69 D, indicates that it is an elongated, asymmetric molecule composed of two domains connected by a short linker (Muller et al., 1994; Muller et al., 1996; Harlos et al., 1994). Each domain is composed of a common core of a three-stranded and a four-stranded β-sheet domain, each domain having similar mobility. Banner et al. (1996) have analyzed the crystal structure of the TF-VIIa complex (Figure 1-1). VIIa binds TF along a "binding stripe" along the length of TF, burying approximately 1800 $D^2$ of surface area. Three major contact points form between TF and VIIa. The N-terminal Gla domain of VIIa binds the C-terminal domain 2 of TF, the interdomain region of TF binds VIIa EGF-1 domain and finally the TF domain 1 region binds the VIIa EGF-2 and protease domains (Banner et al., 1996; Banner, 1997). Key residues for binding identified in the crystal complex correlate well with those identified by site directed mutagenesis (Edgington et al., 1997). The crystal structure analysis supports data gathered by low resolution methods. X-ray and neutron scattering (Ashton et al., 1995) and analytical ultracentrifugation and fluorescence anisotropy (Waxman et al., 1993) previously indicated that VIIa alone and

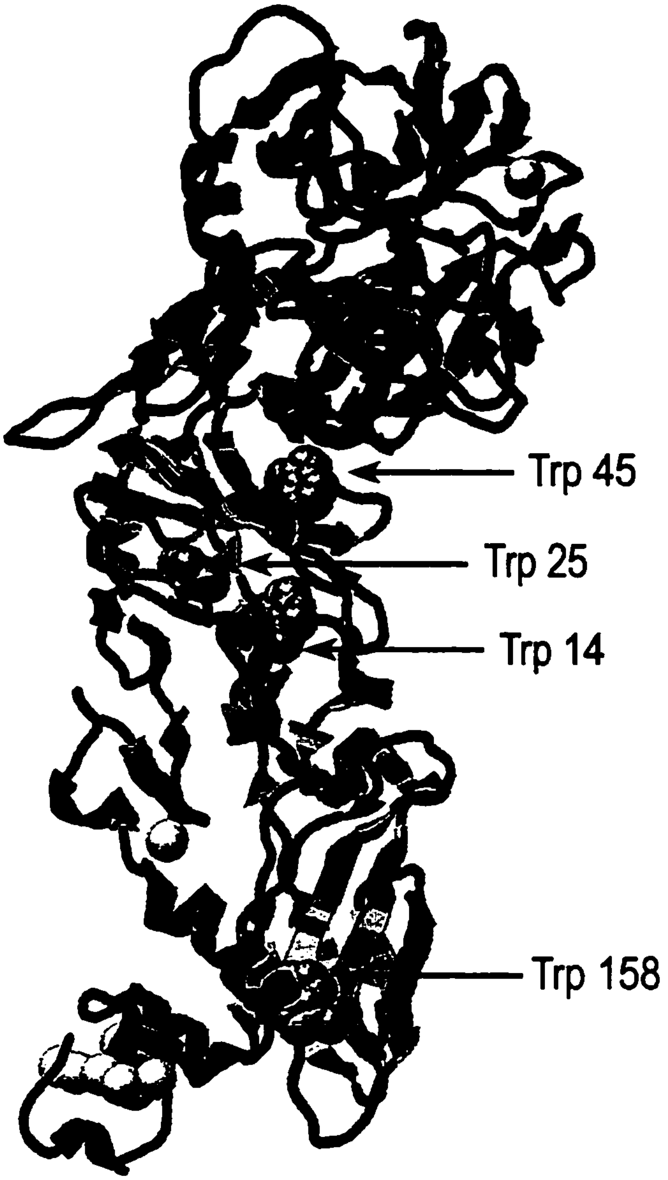


Figure 1-1: Rasmol view of the sTF:VIIa complex based on the 2.0 Å resolution x-ray crystal structure by Banner et al. (1996). The light gray ribbon indicates the main chain of sTF, and the four Trp side chains of sTF are shown as space-filling representations. The dark gray ribbon represents the light and heavy chains of VIIa, and the bound calcium atoms are represented as white spheres. There are seven calcium atoms bound to the Gla domain, one to the EGF domain, and one to the catalytic domain of VIIa.

VIIa in complex with sTF forms an elongated, ellipsoid structure. Furthermore, the dimensions for the complex calculated by these methods are consistent with those calculated from the crystal structure (reviewed in Kirchhofer & Nemerson, 1996).

## Goals of This Study:

The goals of this study are two-fold; first, the development of 5F-Trp as spectroscopic probe and second, the establishment of the fluorescence and $^{19}$F NMR characteristics of 5F-Trp substituted sTF to facilitate future studies characterizing the 5F-Trp sTF-VIIa complex. Development of 5F-Trp as a Trp analog for fluorescence and $^{19}$F NMR studies is desirable in order to broaden the range of proteins, particularly in complexes with other proteins or DNA, which can be studied. These techniques have the additional benefit of allowing observation under solution conditions. Furthermore, fluorescence techniques allow determination of protein structures at physiological concentrations.

sTF and VIIa have been found to form a stoichiometric complex (Waxman et al., 1992). Bach et al. (1986) and Sørensen et al. (1997) found that incorporation of an active site inhibitor of VIIa increases affinity of VIIa for TF. Nemerson and Gentry (1986) observed that activation of VIIa is dependent upon the presence of TF in stoichiometric amounts. Based upon these observations, they formulated the essential activation model that states that VIIa must first bind TF to form an active complex. Substrate may then be bound. The product is then released, leaving the active complex. Using fluorescence energy transfer, McCallum et al. (1996) observed that the VIIa protease domain rotates upon binding TF. However, the crystal structure of the complex indicates that TF does not undergo changes in structure of the sTF backbone, suggesting that TF acts as a scaffold for VIIa to bind (Banner et al., 1996). It remains unknown, however, if TF residues undergo subtler rearrangements that serve to enhance activation of VIIa. The sensitivity of fluorescence and $^{19}$F NMR spectroscopies to local environment provide a tool to observe if these subtler changes occur. Here, the characteristics of the 5F-Trp substituted sTF fluorescence and $^{19}$F NMR spectra are presented with the future intent of characterizing changes in these spectra upon binding VIIa.

## REFERENCES:

Ashton, A.W., G. Kemball-Cook, D.J.D. Johnson, D.M.A. Martin, D.P. O'Brien, E.G. Tuddenham, and S.J. Perkins. 1995. Factor VIIa and the extracellular domains of human tissue factor form a compact complex: a study by X-ray and neutron solution scattering. *FEBS Lett.* 374:141-146.

Bach, R., R. Gentry, and Y. Nemerson. 1986. Factor VII binding to tissue factor in reconstituted phospholipid vesicles: induction of cooperativity by phosphatidylserine. *Biochemistry* 25:4007-4020.

Bach, R. and D.B. Rifkin. 1990. Expression of Tissue Factor Procoagulant Activity: Regulation by Cytosolic Calcium. *Proceedings of the National Academy of Science, USA* 87:6995-6999.

Banner, D.W. 1997. The Factor VIIa / Tissue Factor Complex. *Thromb. Haemost.* 78:512-515.

Banner, D.W., A. D'Arcy, C. Chene, F.K. Winkler, A. Guha, W.H. Konigsberg, Y. Nemerson, and D. Kirchhofer. 1996. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor [see comments]. *Nature* 380:41-46.

Barlati, S. and O. Ciferri. 1970. Incorporation of 5-Methyl- and 5-Hydroxy-Tryptophan into the Protein of *Bacillus subtilis*. *J. Bacteriol.* 101:166-172.

Bronskill, P.M. and J.T. Wong. 1988. Suppression of fluorescence of tryptophan residues in proteins by replacement with 4-fluorotryptophan. *Biochem. J.* 249:305-308.

Danielson, M.A. and J.J. Falke. 1996. Use of 19F NMR to Probe Protein Structure and Conformational Changes. In Annual Review of Biophysics and Biomolecular Structure. R.M. Stroud, W.L. Hubbel, W.K. Olson, and M.P. Sheetz, editors. Annual Reviews, Inc. Palo Alto. 163-195.

Drake. T.A., J. Cheng, A. Chang, and F.B. Taylor. 1993. Expression of Tissue Factor. Thrombomodulin, and E-Selectin in Baboons with Lethal *Escherichia coli* Sepsis. *American Journal of Pathology* 142:1457-1470.

Edgington, T.S., C.D. Dickinson, and W. Ruf. 1997. The Structural Basis of Function of the TF-VIIa Complex in the Cellular Initiation of Coagulation. *Thromb. Haemost.* 78:401-405.

Edgington, T.S., N. Mackman, K. Brand, and W. Ruf. 1991. The structural biology of expression and function of tissue factor. *Thromb. Haemost.* 66:67-79.

Fiore, M.M., P.F. Neuenschwander, and J.H. Morrissey. 1994. The biochemical basis for the apparent defect of soluble mutant tissue factor in enhancing the proteolytic activities of factor VIIa. *J. Biol. Chem.* 269:143-149.

Gerig, J.T. 1994. Fluorine NMR of Proteins. *Prog. NMR Spectrosc.* 26:293-370.

Harlos, K., D.M. Martin, D.P. O'Brien, E.Y. Jones, D.I. Stuart, I. Polikarpov, A. Miller, E.G. Tuddenham, and C.W. Boys. 1994. Crystal structure of the extracellular region of human tissue factor [published erratum appears in Nature 1994 Oct 20;371(6499): 720]. *Nature* 370:662-666.

Haskel, E.J., S.R. Torr, K.C. Day, M.O. Palmier, T.C. Wun, B.E. Sobel, and D.R. Abendschein. 1991. Prevention of arterial reocclusion after thrombolysis with recombinant lipoprotein-associated coagulation inhibitor [see comments]. *Circulation* 84:821-827.

Hasselbacher, C.A., R. Rusinova, E. Rusinova, and J.B.A. Ross. 1995. Spectral Enhancement of Recombinant Proteins with Tryptophan Analogs: The Soluble Domain of Human Tissue Factor. In Techniques in Protein Chemistry. J.W. Crabb, editor. Academic Press, New York. 349-356.

Hogue, C.W. and A.G. Szabo. 1993. Characterization of aminoacyl-adenylates in B. subtilis tryptophanyl-tRNA synthetase, by the fluorescence of tryptophan analogs 5-hydroxytryptophan and 7-azatryptophan. *Biophys. Chem.* 48:159-169.

Hogue, C.W.V. 1994. Tryptophanyl-tRNA Synthetase and Its Role in the Incorporation of New Intrinsic Fluorescent Probes into Proteins. Dissertation, University of Ottawa. Ottawa, Canada.

Hott, J.L. and R.F. Borkman. 1989. The non-fluorescence of 4-fluorotryptophan. *Biochem. J.* 264:297-299.

Kirchhofer, D. and Y. Nemerson. 1996. Initiation of blood coagulation: the tissue factor/factor VIIa complex. *Curr. Opin. Biotechnol.* 7:386-391.

Lark, K.G. 1969. Incorporation of 5-Methyltryptophan into the Protein of *Escherichia coli* 15 T-(555-7). *J. Bacteriol.* 97:980-982.

Lau, E.Y. and J.T. Gerig. 1997. Effects of Fluorine Substitution on the Structure and Dynamics of Complexes of Dihydrofolate Reductase (*Escherichia coli*). *Biophysical Journal* 73:1579-1592.

McCallum, C.D., R.C. Hapak, P.F. Neuenschwander, J.H. Morrissey, and A.E. Johnson. 1996. The location of the active site of blood coagulation factor VIIa above the membrane surface and its reorientation upon association with tissue factor. A fluorescence energy transfer study. *J. Biol. Chem.* 271:28168-28175.

Mueller, B.M., R.A. Reisfeld, T.S. Edgington, and W. Ruf. 1992. Expression of tissue factor by melanoma cells promotes efficient hematogenous metastasis. *Proc. Natl. Acad. Sci. U. S. A.* 89:11832-11836.

Muller, Y.A., M.H. Ultsch, and A.M. de Vos. 1996. The crystal structure of the extracellular domain of human tissue factor refined to 1.7 A resolution. *J. Mol. Biol.* 256:144-159.

Muller, Y.A., M.H. Ultsch, R.F. Kelley, and A.M. de Vos. 1994. Structure of the extracellular domain of human tissue factor: location of the factor VIIa binding site. *Biochemistry* 33:10864-10870.

Nemerson, Y. 1988. Tissue factor and hemostasis [published erratum appears in Blood 1988 Apr;71(4):1178]. *Blood* 71:1-8.

Nemerson, Y. 1995. Tissue factor: then and now. *Thromb. Haemost.* 74:180-184.

Nemerson, Y. and R. Gentry. 1986. An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage [published erratum appears in Biochemistry 1987 Feb 10;26(3):974]. *Biochemistry* 25:4020-4033.

Neuenschwander, P.F. and J.H. Morrissey. 1995. Alteration of the substrate and inhibitor specificities of blood coagulation factor VIIa: importance of amino acid residue K192. *Biochemistry* 34:8701-8707.

Østerud, B. 1997. Tissue Factor: a complex biological role. *Thromb. Haemost.* 78:755-758.

Paborsky, L.R., I.W. Caras, K.L. Fisher, and C.M. Gorman. 1991. Lipid association, but not the transmembrane domain, is required for tissue factor activity. Substitution of the transmembrane domain with a phosphatidylinositol anchor. *J. Biol. Chem.* 266:21911-21916.

Pardee, A.B., V.G. Shore, and L.S. Prestidge. 1956. Incorporation of Azatryptophan into Proteins of Bacteria and bacteriophage. *Biochim. Biophys. Acta* 21:406-407.

Pratt, F.A. and C. Ho. 1975. Incorporation of Fluorotryptophan in Proteins of *Escherichia coli*. *Biochemistry* 14:3035-3040.

Robertson, D.E., P.A. Kroon, and C. Ho. 1977. Nuclear Magnetic Resonance and Fluorescence Studies of Substrate-induced Conformational Changes of Histidine-binding Protein J of *Salmonella typhimurium*. *Biochemistry* 16:1443-1451.

Ross, J.B.A., A.G. Szabo, and C.W.V. Hogue. 1997. Enhancement of Protein Spectra with Tryptophan Analogs: Fluorescence Spectroscopy of Protein-Protein and Protein-Nucleic Acid Interactions. In Fluorescence Spectroscopy. L. Brand and M.L. Johnson, editors. Academic Press, New York. 151-190.

Röttingen, J.A., T. Enden, E. Camerer, J.G. Iversen, and H. Prydz. 1995. Binding of human factor VIIa to tissue factor induces cytosolic Ca2+ signals in J82 cells, transfected COS-1 cells, Madin-Darby canine kidney cells and in human endothelial cells induced to synthesize tissue factor. *J. Biol. Chem.* 270:4650-4660.

Ruf, W. and T.S. Edgington. 1994. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. *FASEB J.* 8:385-390.

Schlesinger, S. 1968. The Effect of Amino Acid Analogues on Alkaline Phosphatase Formation in *Escherichia coli* K-12. *J. Biol. Chem.* 243:3877-3883.

Semeraro, N. and M. Colucci. 1997. Tissue Factor in Health and Disease. *Thromb. Haemost.* 78:759-764.

Shen, F., S.J. Triezenberg, P. Hensley, D. Porter, and J.R. Knutson. 1996. Transcriptional activation domain of the herpesvirus protein VP16 becomes conformationally constrained upon interaction with basal transcription factors. *J. Biol. Chem.* 271:4827-4837.

Sørensen, B.B., E. Persson, P.-O. Freskgard, M. Kjalke, M. Ezban, T. Williams, and L.V.M. Rao. 1997. Incorporation of an active site inhibitor in factor VIIa alters the affinity for Tissue Factor. *J. Biol. Chem.* 272:11863-11868.

Taubman, M.B., J.T. Fallon, A.D. Schecter, P. Giesen, M. Mendlowitz, B.S. Fyfe, J.D. Marmur, and Y. Nemerson. 1997. Tissue Factor in the Pathogenesis of Atherosclerosis. *Thromb. Haemost.* 78:200-204.

Waxman, E., W.R. Laws, T.M. Laue, Y. Nemerson, and J.B.A. Ross. 1993. Human factor VIIa and its complex with soluble tissue factor: evaluation of asymmetry and conformational dynamics by ultracentrifugation and fluorescence anisotropy decay methods. *Biochemistry* 32:3005-3012.

Waxman, E., J.B.A. Ross, T.M. Laue, A. Guha, S.V. Thiruvikraman, T.C. Lin, W.H. Konigsberg, and Y. Nemerson. 1992. Tissue factor and its extracellular soluble domain: the relationship between intermolecular association with factor VIIa and enzymatic activity of the complex. *Biochemistry* 31:3998-4003.

Wilcox, J.N., K.M. Smith, S.M. Schwartz, and D. Gordon. 1989. Localization of Tissue Factor in the Normal Vessel Wall and in the Athersclerotic Plaque. *Proceedings of the National Academy of Science, USA* 86:2839-2843.

Wong, C.Y. and M.R. Eftink. 1997. Biosynthetic incorporation of tryptophan analogues into staphylococcal nuclease: effect of 5-hydroxytryptophan and 7-azatryptophan on structure and stability. *Protein Sci.* 6:689-697.

Xu, Z.J., M.L. Love, L.Y. Ma, M. Blum, P.M. Bronskill, J. Bernstein, A.A. Grey, T. Hofmann, N. Camerman, and J.T. Wong. 1989. Tryptophanyl-tRNA synthetase from Bacillus subtilis. Characterization and role of hydrophobicity in substrate recognition. *J. Biol. Chem.* 264:4304-4311.

Zioncheck, T.F., S. Roy, and G.A. Vehar. 1992. The cytoplasmic domain of tissue factor is phosphorylated by a protein kinase C-dependent mechanism. *J. Biol. Chem.* 267:3561-3564.

# CHAPTER 2

## $^{19}$F NMR of 5-Fluorotryptophan in Soluble Human Tissue Factor: Comparison with Results from Tryptophan Fluorescence

from Zemsky et al., manuscript submitted

## INTRODUCTION

Following tissue damage, the extrinsic pathway of the blood coagulation cascade is initiated by the interaction between Tissue Factor (TF), a membrane-bound glycoprotein, and Factor VII/VIIa (VII/VIIa), a serine protease that circulates in the blood (see reviews by Bach, 1988; Nemerson, 1988, 1995; Edgington et al., 1991; Davie, 1995; Østerud, 1997). VII is a 406-residue, single-chain zymogen. The active enzyme, VIIa, is generated by a specific internal cleavage, forming a 152-residue light chain and a 254-residue heavy chain. TF, a member of the cytokine receptor superfamily, is a 263-residue polypeptide that consists of an extracellular domain (residues 1-219), a single transmembrane domain (residues 243-263), and a cytoplasmic domain (residues 243-263). In the absence of TF, VIIa has virtually no proteolytic activity towards its protein substrates, Factor IX (IX) and Factor X (X). Thus, with respect to the coagulant activity of VIIa, binding to TF is obligatory, and thus TF serves as an essential cofactor for VIIa (Nemerson & Gentry, 1986).

The association between TF and VIIa occurs at a membrane surface, involving protein-protein and protein-lipid interactions. To investigate the features of the protein-protein interaction, we have used the soluble, truncated extracellular domain of TF (sTF), expressed in *Escherichia coli* (*E. coli*). Although sTF lacks the transmembrane and cytoplasmic domains, it retains procoagulant activity when phospholipids are present (Waxman et al., 1992; Neuenschwander & Morrissey, 1992; Fiore et al., 1994). It has been shown that in the absence of lipids, sTF and VIIa form a high- affinity, 1:1 complex with an equilibrium dissociation constant, at 20°C, of about 1 nM (Waxman et al., 1992). Hydrodynamic studies by analytical ultracentrifugation and time-resolved fluorescence anisotropy (Waxman et al., 1993a), as well as by x-ray and neutron scattering (Ashton et al., 1995), have shown that both proteins are highly asymmetric. Ashton et al. also show that a major conformational change occurs in VIIa upon binding sTF, specifically a loss in the segmental flexibility of the protease domain.

The recent x-ray crystal structure of the complex between sTF and inhibited VIIa by Banner et al. (1996) reveals important details of the asymmetry and the intermolecular interaction. The $\gamma$-carboxy glutamic acid-rich (Gla) domain, two epidermal growth factor (EGF) domains, and serine protease domain of VIIa all interact more with the two domains of sTF than with each other (see Figure 1-1), burying approximately 1800 $Å^2$ of surface between the two proteins. In addition, comparison of the conformations of the main chain $\alpha$ carbons of free sTF, obtained from the crystal structure by Muller et al. (1994), with those of sTF bound to VIIa, shows no significant differences ($\Delta$ r.m.s. = 0.5 Å) in the main chain conformation of sTF (Banner et al., 1996). This would suggest that the extracellular domain of TF provides a template to stabilize the catalytically efficient conformation of VIIa, and that conformational changes, which might occur in sTF upon association with VIIa, are restricted mainly to rearrangements and possible motions of side chains.

Similar to full-length TF (Sorensen et al., 1997; Bach et al., 1986), the affinity between sTF and VIIa is enhanced when the active site of VIIa is occupied by a covalently bound peptide inhibitor (Waxman, 1993). Thus, substrate and TF (or sTF) binding by VIIa are thermodynamically linked, as predicted by the ordered addition, essential activation model for the TF-initiated pathway of blood coagulation (Nemerson & Gentry, 1986). The allosteric interdependence of TF and substrate binding suggests conformational differences between the binary cofactor:enzyme complex and the ternary complexes that include substrate (such as IX or X) or an inhibitory peptide.

To elucidate the subtle conformational changes that might occur when sTF interacts with VIIa, we are using $^{19}$F NMR spectroscopy. $^{19}$F NMR is particularly sensitive to changes in the local electrostatic fields and changes in van der Waals contacts. This makes it particularly useful for observation of subtle changes in structure in different locations within fluorine-labelled proteins with molecular weights up to 100,000 (Gerig, 1994; Danielson & Falke, 1996). The respective molecular weights of sTF and its complex with VIIa are about 25,000 and 74,000 (Waxman et al., 1993a), within the molecular weight range accessible to $^{19}$F NMR. To study protein-protein complexes, selective labeling of one protein with fluorinated amino acids allows observation of conformational changes that occur in the local environment of the fluorine reporter group while the partner protein remains "silent". Selective labeling of recombinant proteins expressed in *E. coli* can be achieved biosynthetically, for example using mono-fluorinated tryptophan (Trp) or phenylalanine (Phe) analogs and the appropriate auxotrophic cells (Danielson & Falke, 1996). It is important that the amino acid analogs be incorporated at sites that are sensitive to interaction. For ease of interpretation of the NMR spectra, it also is desirable to replace the minimum number of residues.

The x-ray crystal structure of the sTF:VIIa:inhibitor complex (Banner et al., 1996) shows that two of the four Trp residues in sTF, Trp 45 and Trp 158, are involved in direct intermolecular interactions with VIIa. First, Trp 45 forms an amide hydrogen bond with the carbonyl oxygen of Phe 275 of the VIIa protease domain; this is the only main chain-main chain contact for the two proteins. In addition, the indole ring of Trp 45 packs against the side chain of Arg 277 of VIIa. Second, Trp 158 is involved in the main hydrophobic interaction with VIIa, which includes participation from the nearby Cys 186 – Cys 209 disulfide bridge and Val 207 of sTF to Phe 31 in the Gla domain of VIIa. Thus, the Trp residues of sTF, replaced by F-Trp analogs, should be suitably sited probes for studying the interaction with VIIa, provided the resonances of the four F-Trp residues can be resolved and assigned.

The $^{19}$F NMR spectroscopy and functional properties of sTF labeled with 5-fluoro-Trp (5F-Trp) have been investigated. The resonance assignments for each 5F-Trp residue are based on differences between the $^{19}$F NMR spectra of wild type and single-Trp replacement mutants. Also, paramagnetic line broadening experiments were carried out to assess the solvent accessibility of the 5F-indole side chains. The $^{19}$F NMR data for the wild type and mutant proteins are compared with those from a previous investigation based on Trp absorbance and fluorescence spectroscopy (Hasselbacher et al., 1995a), showing that the NMR and optical spectroscopies provide complementary information. In particular, the 5F-Trp $^{19}$F NMR spectra reveal subtle perturbations in the local environment of the NMR probe that result from single Trp-to-Phe mutations within the same protein domain. The sensitivity of $^{19}$F NMR to subtle structural perturbations, demonstrated from the mutant spectra, suggests that $^{19}$F NMR will be a valuable tool for investigating the macromolecular interactions in the binary sTF:VIIa complex and ternary complexes with substrates or inhibitors.

## MATERIALS AND METHODS

### Reagents

5-d,l-Fluorotryptophan was purchased from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI) and Acros (Pittsburgh, PA). Recombinant human VIIa was a generous gift from Novo Nordisk (Denmark), and factor X was purified from human plasma by the methods of Miletich, et al. (1981) and Broze & Majerus (1980). 1,2-Dioleoyl-*sn*-glycero-3-phosphotidylserine and 1,2-dioleoyl-*sn*-glycero-3-phophotidylcholine were obtained from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate Spectrozyme was obtained from American Diagnostica Inc. (Greenwich, CT). Guanidinium chloride (Gdm·Cl) was purchased from Heico Chemicals (Delaware Water Gap, PA). Gadolinium (Gd) and diethylenetriamine-pentaacetic acid:gadolinium (III) dihydrogen salt hydrate (Gd:DPTA) complex were obtained from Aldrich (Milwaukee, WI).

### Expression and purification of wild type and mutant sTF with 5F-Trp incorporation

The wild type and mutant plasmids (W14F, W45F, W158F) were the same as previously described (Hasselbacher et al., 1995a). Following standard protocols (Sambrook et al., 1989), the plasmids were transformed into the *E. coli* tryptophan auxotroph CY15077 EA2 (Ross et al., 1997; Hasselbacher et al., 1995). Then the resultant strains were grown overnight at 37°C in 6 × 1 L of Terrific broth supplemented with 4 ml/L of glycerol. The following morning, the cells were spun down, each pellet from 1 L of culture medium was washed in 100 ml of M9 salts, and then resuspended in 1 L of M9 salts, supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.04% glucose, 1% casamino acids, 0.1% thiamine and 60 μg/ml ampicillin. The cultures were shaken for 1 hour at 37°C, then transferred to 30°C, supplemented with 60 mg/L 5F-Trp, and shaken for an additional 30 minutes. Isopropyl-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce protein expression, and after 4-5 hours of shaking, the cells were harvested by centrifugation.

Previously, we purified sTF and the single Trp replacement mutants (Trp-to-Phe) from concentrated media after overnight induction at 20°C (Hasselbacher et al., 1995a). With the shorter induction period used here, most of the expressed protein is retained in the cells. Using the following modification, the proteins were released from the periplasmic fraction by osmotic shock lysis. The cell pellets were resuspended in 1 L of 30 mM Tris, pH 8, 20% sucrose, 1 mM EDTA, and stirred for 5 minutes at room temperature. The cells were spun down and resuspended in 1 L of ice-cold 5 mM $MgSO_4$ and stirred for 5 minutes at 4°C (adapted from Snavely et al., (1989)). The cells were pelleted and discarded, and $(NH_4)_2SO_4$ was added to the supernatant to 65% saturation. The remaining steps for purification were as described by Waxman et al. (1992).

### Absorption spectra and LINCS analysis

Absorption spectra were measured between 260 and 340 nm, at room temperature, using a dual beam Hitachi U-3210 spectrophotometer. Sample buffer was TBS (50 mM

Tris, 100 mM NaCl, 1mM EDTA, and 1 mM $NaN_3$, pH 7.4). Spectra were obtained for each sample: native in TBS and denatured in 6M Gdm·Cl. The percentage incorporation of 5F-Trp was calculated from the denatured absorbance spectrum by analysis as a linear combination of spectra (LINCS) (Waxman et al., 1993b) using as the basis set the absorbance spectra of N-acetyl-tryptophanamide (NATrpA), N-acetyl-tyrosinamide (NATyrA), and 5F-Trp, also in 6 M Gdm·Cl. The wavelength region used for fitting the absorbance spectra of the denatured proteins was between 340 and 270 nm to avoid interference from phenylalanine absorption. The low extinction contributions from the two disulfide bridges were ignored. Thus, the fit essentially should represent the absorption of Tyr, Trp and 5F-Trp in the denatured protein spectra. The quality of the fits was evaluated by the decrease in the $\chi^2$ statistic when changing from two basis spectra (NATyrA and 5F-Trp) to three (NATyrA, NATrpA and 5F-Trp). The criterion for a satisfactory analysis was recovery of the correct mol ratio of Tyr to total 'Trp' ('Trp' = Trp + 5F-Trp) in the protein. The percentage of 5F-Trp incorporated was determined from the mol ratio of 5F-Trp to total 'Trp'.

**Mass spectrometry**

Electrospray mass spectrometry (ESMS) and liquid chromatography ESMS (LC ESMS) were performed at the W. Alton Jones Cell Science Center.

To estimate the percent of 5F-Trp incorporation using the mass spectrometry data, the peak for each species, with all, some, or none of the Trp residues replaced by 5F-Trp, was identified by its appropriate mass. Higher mass peaks resulting from salt adducts were ignored. In order to obtain the mol fraction of each species in the total protein, the peak height for each appropriate mass was divided by the sum of the identified peak heights, and multiplied by their respective mol fraction of 5F-Trp to yield the fractional incorporation of 5F-Trp represented by each species. The sum of these values for each protein was used as the estimate of percent 5F-Trp incorporation.

### Cofactor activity

Wild type and mutant sTF proteins, with or without 5F-Trp, were assayed for cofactor activity as previously described by Hasselbacher et al. (1995a). Briefly, the rate of production of Xa at a fixed concentration of VIIa and saturating concentration of substrate X was measured as a function of cofactor concentration. The concentration of cofactor formed, [cofactor:VIIa], is proportional to the observed velocity. The relative catalytic efficiency, $k_{cat}$, of each cofactor:VIIa complex was obtained directly by comparison of the maximum velocity of Xa production with that of unlabeled wild type cofactor. Apparent equilibrium dissociation constants, $K_d$, were obtained from fitting each titration curve by least squares, using the appropriate root of the quadratic equation

$$[\text{cofactor:VIIa}]^2 - [\text{cofactor:VIIa}](K_d + [\text{VIIa}_o] + [\text{cofactor}_o]) + [\text{VIIa}_o][\text{cofactor}_o] = 0$$

where $[\text{VIIa}_o]$ is the total concentration of enzyme, and $[\text{cofactor}_o]$ is the total concentration of wild type or mutant sTF with or without 5F-Trp.

## $^{19}$F NMR

Magnetic resonance shifts were assigned for each Trp residue by comparing spectra of single Trp replacement mutants with the wild type protein spectrum. $^{19}$F NMR spectra were obtained at 470 MHz on a Varian Unity 500 at 25°C with a triple resonance probe with the center proton coil tuned to fluorine. Standard coupled parameters were 12 MHz spectral width, 16 K data points, 60-degree pulse-width and a relaxation delay of 0.5 seconds, and processing parameters included either 25 or 10 Hz line broadening. The protein samples for NMR ranged between 2.5 mg/ml and 20 mg/ml, and were in TBS buffer with 10% $D_2O$ (v/v) as the lock solvent. 3F Phenylalanine (3F-Phe) was used as an external standard (-38.0 ppm relative to trifluoroacetic acid). Stock solutions of 100 mM $GdCl_3$/500 mM EDTA or 100 mM Gd:DPTA (both adjusted to pH 7.1) were used for the line broadening experiments, which were performed by sequential addition of the gadolinium complexes to appropriate concentrations.

## RESULTS AND DISCUSSION

### 5-Fluoro-d,l-tryptophan: protein expression, incorporation, and effects on function

The yields of proteins expressed in the *E. coli* Trp auxotroph CY15077 EA2 (Ross et al., 1997; Hasselbacher et al., 1995b) varied in a mutant-dependent manner. For example, the yields from 6 L of bacterial culture were about 65 mg for wild type sTF but only about 1 mg for mutant W14F and much less for W25Y. Unfortunately, the quantity of W25Y recovered was insufficient for NMR. It should be noted that the same levels of protein expression were observed, whether Trp (Hasselbacher et al., 1995a) or 5F-Trp (this study) was used for protein synthesis. The relatively low expression of W14F and W25Y suggests that replacement of these Trp residues with amino acids that have smaller side chains interferes with protein folding. According to the x-ray crystal (Muller et al., 1994; Harlos et al., 1994), Trp 14 and Trp 25 are essentially buried within the protein matrix of Domain I. In addition, Trp 25 is at the center of a pattern of extensive packing interactions, characteristic of the fibronectin III family (Muller et al., 1996). Moreover, both the absorption and fluorescence spectra of Trp 14 and Trp 25 indicate that their indole side chains are in tightly constrained environments (Hasselbacher et al., 1995a).

Incorporation of F-Trp analogs is generally less than 100%, depending upon the analog and method of incorporation (Danielson & Falke, 1996). Two approaches were used to assess the level of 5F-Trp incorporation into wild type and mutant sTF proteins. The first involved fitting absorbance spectra of wild type and mutant proteins, denatured by 6 M Gdm·Cl, by LINCS analysis (Waxman et al., 1993b) using NATrpA, NATyrA and 5F-Trp as basis spectra. According to this method, 5F-Trp incorporation varied from 77% to 100% percent, depending upon the protein (Table 1). As indicated in the Materials and Methods section, the criterion for a satisfactory LINCS analysis is a minimum $\chi^2$ statistic and a correct Tyr to total Trp (Trp + 5F-Trp) ratio. For example, using only two basis set spectra to generate the fit for 5F-Trp W14F-sTF, either NATrpA and NATyrA (shown in Figure 2-1) or 5F-Trp and NATyrA, results in incorrect Tyr to total Trp ratios that are either 58% too low or 24% too high, respectively. Using NATrpA, NATyrA and 5F-Trp together as the basis spectra, instead of either NATrpA and NATyrA alone or 5F-Trp and NATyrA alone, reduces the $\chi^2$ statistic by 93% or 37%, respectively. In addition, when all three basis spectra are used to fit the spectrum, the Tyr to total Trp ratio is only 2% greater than the theoretical value. On the other hand, for the LINCS analysis of the wild type protein, the $\chi^2$ statistic decreases by less than 1% when NATrpA is added as the third basis spectrum. Also, the correct Tyr to total Trp ratio is obtained when the basis set includes only 5F-Trp and NATyrA. Thus, according to LINCS analysis, 5F-Trp incorporation in the wild type protein is essentially 100%.

The second approach used to examine the level of 5F-Trp incorporation was by mass spectrometry. It is possible that for any protein examined that all, some, or none of the Trp residues were replaced with 5F-Trp. The wild type protein spectrum, shown in Figure 2-2A, has a major peak located at the mass expected for sTF with all four Trps replaced by 5F-Trp. A minor peak is located at the mass expected for sTF with three of the four Trps replaced by 5F-Trp. These data indicate efficient replacement of Trp by 5F-Trp, essentially in agreement with LINCS analysis. Also in agreement with LINCS analysis, the mass spectrometry data shows that replacement of Trp by 5F-Trp was less efficient in the Trp-to-Phe mutants. Moreover, the mass spectrum provides a direct assessment of micro-heterogeneity by revealing proteins where all, some, or none of the Trp residues are replaced by 5F-Trp. For example, the mass spectrum for 5F-Trp W14F, shown in Figure 2-2B, demonstrates that the expressed protein is a mixture of such species. On this basis, for example, the estimates from mass spectrometry for 5F-Trp incorporation for wild type and W14F sTF are 93% and 74%, respectively. Inspection of Table 1 shows that the estimates of analog incorporation obtained from the mass spectrum, simply by comparing relative peak heights, are the same within experimental error (3% to 10%) as those determined from the absorption spectrum of the Gdm·Cl denatured protein by using LINCS analysis.

Cofactor activation of VIIa by 5F-Trp labeled wild type and mutant proteins, as measured by chromogenic assay, was essentially the same as that previously reported for the corresponding non-analog containing proteins (Hasselbacher et al., 1995a). The apparent $K_d$ values for the wild type and W158F were between 1 and 2 nm and the values for W14F andW25Y were approximately 4 nm. These values correlated well with the previous study. In contrast, the apparent $K_d$ for W45F (two different preparations) was approximately 100 nm, which was an order of magnitude higher than previously reported. This affect can be shown to be due to the weaker binding previously shown to be caused by poor VIIa preparations supplied by Novo Nordisk. The maximum catalytic activities of the VIIa complexes with mutant or wild type cofactors were indistinguishable. Thus, the replacement of Trp residues with 5F-Trp in sTF does not introduce functional perturbations.

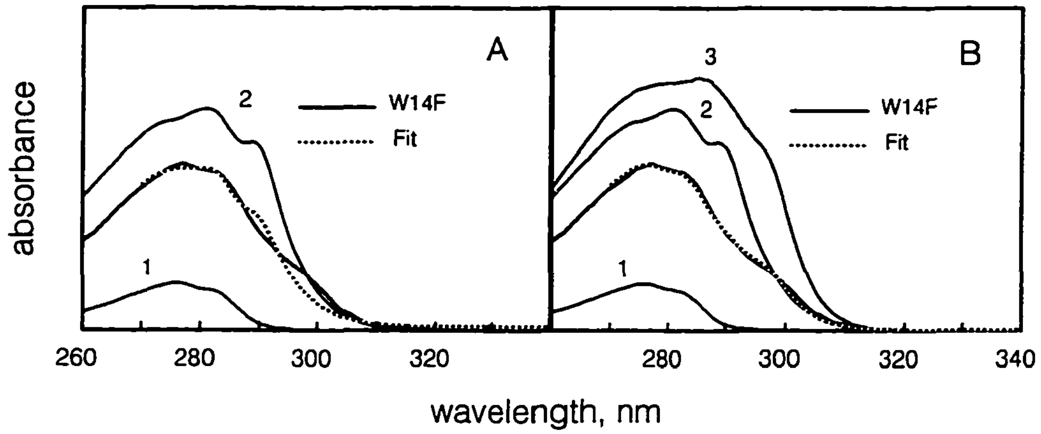


Figure 2-1: LINCS analyses of denatured W14F (see **Materials and Methods**) using either absorbance spectra of (A) NATyrA (curve 1) and NATrpA (curve 2) or (B) NATyrA (curve 1), NATrpA (curve 2), and 5F-Trp (curve 3) as basis sets. The protein absorbance spectrum was analyzed from 340 to 270 nm, and the dotted lines represent the "best fits" using each basis set.

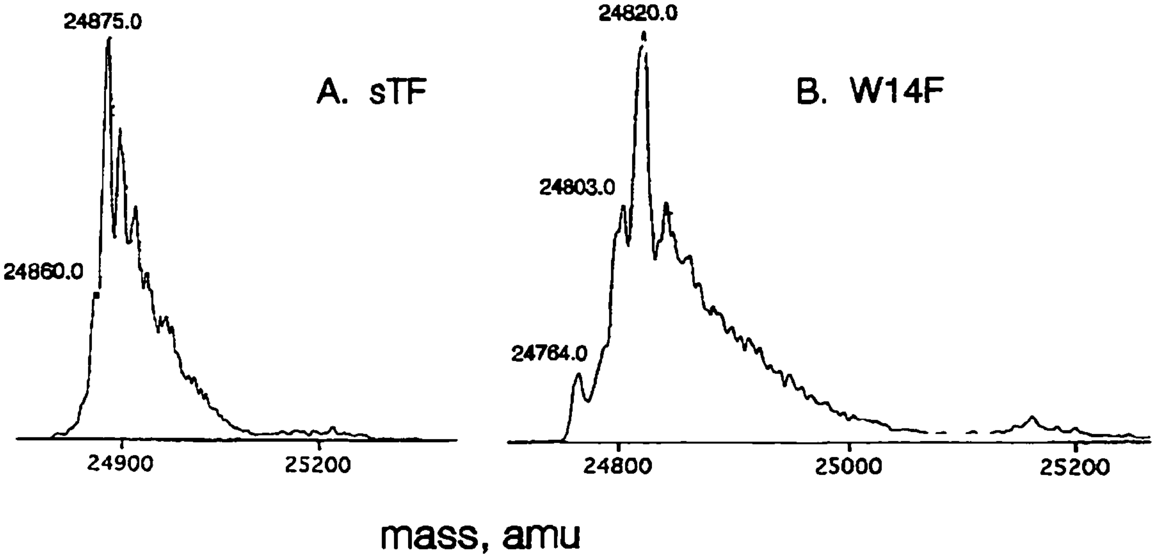


Figure 2-2: Mass spectra of (A) wild type and (B) W14F sTF.

Table 1. Percent 5F-Trp incorporation in mutant and wild type sTF as estimated by LINCS analysis and by mass spectrometry.

| Protein | LINCS | Mass Spectrum |
|---|---|---|
| wild-type | 100 | 94/100 |
| W14F | 77 | 74 |
| W25Y | 72 | 68/73 |
| W45F | 83 | 73 |
| W158F | 79 | 74 |

Percent incorporation of 5F-Trp in mutant and wild type proteins was determined as described in Materials and Methods. The values represent the percent 5F-Trp incorporated into the Trp sites present in each protein so that for the wild type the value is the percent of the 4 Trp sites present while for the mutants it is the percent of the 3 Trp sites present. Estimated precision of values obtained from LINCS is ± 8 %. Values obtained from mass spectrometry represent results of individual measurements.

### $^{19}$F NMR assignments for 5F-Trp residues in sTF

Resonance peaks were assigned for each Trp residue by comparing the spectrum of wild type 5F-Trp sTF with the individual spectra of the single Trp replacement mutants (Figure 2-3). Four Trp resonances can be identified in the wild type sTF spectrum. There are two well-resolved peaks at -45.39 and -47.21 ppm, and a single peak of two overlapping resonances with maxima at -47.87 and -47.95 ppm. The ratio of the integrals corresponding with the three peaks is 1:1:2, indicating that all four 5F-Trp residues contribute equally to the signal. Observing which peak was eliminated in the spectra of mutants W14F, W45F, and W158F identified the resonance of each 5F-Trp residue. Since the ratio of the integrals of the peaks in the spectra for mutants W14F and W45F were 1:1:1 (Figure 2-3), it was determined that the overlapping resonances must be due to Trp 14 and Trp 45, with the resonance due to Trp 14 being the most down-field near -48 ppm. The W158F spectrum shows two resolved peaks with a ratio of 1:2 for their integrals, and the loss of the central peak at -47.21 ppm indicates that this missing peak correlates with Trp 158. Accordingly, the remaining peak in the most up-field position near -45.4 is due to Trp 25. The ratios of the peak integrals show that replacement of Trp residues by 5F-Trp in the mutants, which had significantly less efficient incorporation of 5F-Trp than wild type, is random.

It should be noted that with the exception of mutant W158F, the resonances of the Domain I 5F-Trp residues in the mutant spectra, especially those of W14F and to a lesser extent those of W45F, are shifted with respect to their wild type frequencies. For example, the W14F spectrum shows shifts of about 0.3 ppm, down-field for the 5F-Trp 25 resonance and up-field for the 5F-Trp 45 resonance. By contrast, the peak associated with 5F-Trp 158

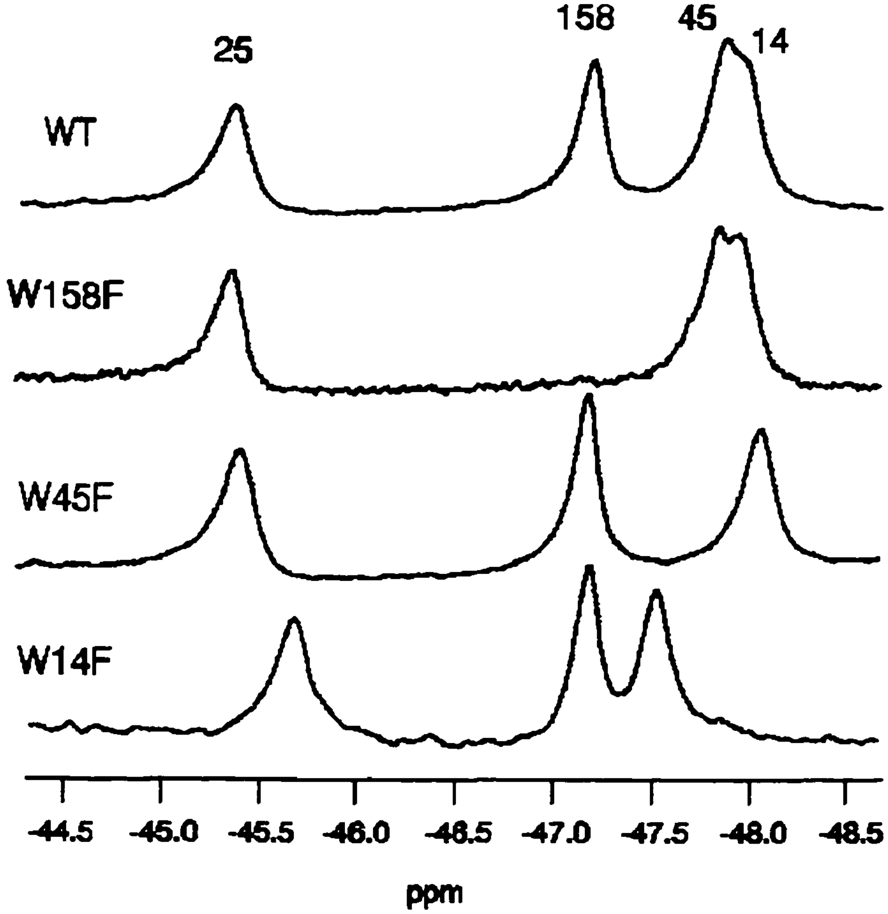


Figure 2-3: $^{19}F$ NMR spectra (470 MHz, proton-decoupled) of wild type, W158F, W45F and W14F sTF, obtained as described in **Materials and Methods**. The sample buffer contained TBS, 1 mM EDTA, 1 mM $NaN_3$, and 10% $D_2O$ as the solvent lock. 3F Phenylalanine was used as an external standard (-38.0 ppm relative to trifluoroacetic acid).

at -47.21 ppm, the only resonance originating from Domain II, is unaffected by the Domain I mutations (Figure2-3).

The chemical shifts of $^{19}$F are influenced strongly by van der Waals contacts and electrostatic interactions (Gerig, 1994; Danielson & Falke, 1996). Accordingly, the perturbations in the chemical shifts of 5F-Trp 25 and 5F-Trp 45 in Domain I mutant spectra suggest that substitution of the indole ring with the smaller volume phenyl ring results in altered packing interactions with adjacent side chains of neighboring residues. In turn, other, more distant side chain packing interactions in Domain I also are affected, including those of the indole groups of 5F-Trp 25 and 5F-Trp 45. This is not unreasonable as the x-ray crystal structures of wild type sTF (Muller et al., 1994; Muller et al., 1996; Harlos et al., 1994) indicate that the indole groups of Trp 25 and Trp 45 are 11 to 13 Å from the indole group of Trp 14. The fact that the single Trp replacement mutants all form functional complexes with VIIa, however, indicates that the structural perturbations of the cofactor matrix are sufficiently subtle that the essential features of the wild type cofactor interaction with VIIa are maintained.

The smaller perturbations in the 5F-Trp resonances associated with Domain I of W45F, compared to those of W14F, are consistent with the fact that unlike Trp 14, Trp 45 is not completely buried within the protein matrix. Instead, Trp 45 is partially solvent exposed and in a less constrained environment than Trp 14 (Hasselbacher et al., 1995a). Thus, replacing the indole ring of Trp 45 with the smaller phenyl ring is not likely to cause as much perturbation of the interior packing interactions. However, the binding of W45F to

VIIa is significantly weaker than the binding of either W14F or wild type sTF, indicating that mutation of Trp 45 perturbs intermolecular side chain interactions. As described by Banner et al. (1996), the indole group of Trp 45 not only forms intermolecular contact with Arg 277 of VIIa, but it also forms a crucial intramolecular contact with Arg 74 in Domain I. The side chain of Arg 74 is involved in a network of intramolecular interactions with other Domain I residues, which together form a hydrophobic pocket that provides a "lock-and-key' intermolecular interaction with Met 306 of VIIa. Compared with indole (or 5F-indole), the phenyl ring has a much smaller surface area for favorable van der Waals interaction and a substantially smaller dipole moment for favorable charge-dipole or dipole-dipole interaction. Thus, replacing the indole ring of Trp 45 with a phenyl ring would be expected to reduce intramolecular interaction with the side chain of Arg 74. This could allow greater mobility of Arg 74 in the unbound mutant. Restricting the mobility of Arg 74 to form the complex would impose an entropic penalty on association, and could account for the weaker affinity of the mutant cofactor:VIIa complex while not substantially affecting catalytic efficiency.

**Paramagnetic line broadening**

Based on the difference absorbance and fluorescence spectra of sTF and the single Trp-replacement mutants (Hasselbacher et al., 1995a), the indole rings of both Trp 45 and Trp 158 are partially exposed to solvent, with that of Trp 45 being the more exposed. By comparison, the indole rings of Trp 14 and Trp 25 are essentially buried in the protein matrix. The relative solvent accessibilities of Trp 45 and Trp 14 could be assessed from fluorescence quenching. Trp 25 and Trp 158, however, are essentially nonfluorescent, and conclusions regarding their local environments were derived on the basis of the spectral shift and resolution of the vibrational bands of each residue's absorption spectrum. Using the specifically located fluorine atom as a reporter group, and observing its interaction with paramagnetic metals, $^{19}$F NMR can be used to obtain more detailed information about solvent exposure. Paramagnetic metals broaden the $^{19}$F NMR spectrum of accessible fluorine atoms via spin-spin relaxation, which has an inverse sixth power distance dependence, becoming effective within distances of a few angstroms. In order to limit accessibility of the paramagnetic metal to only solvent-exposed fluorine atoms, the metal is chelated in different complexes. The Gd:EDTA complex used in this study has a hydrated diameter of 11.7 Å limiting Gd access to only solvent-exposed residues. Furthermore, complexation of Gd with EDTA reduces the dependence of the relaxation on the electronic environment (Luck, 1991).

Accompanying titration of wild type sTF with Gd:EDTA, the resolved resonance of 5F-Trp 158 broadens significantly, as measured by increased full-width at half-height, while the resolved resonance of 5F Trp 25 and the overlapping resonances of 5F-Trp 14 and 5F-Trp 45 appear to be essentially unaffected (Figure2-4A). Gd:EDTA carries a net charge of -1, and as a result may not interact with residues in close proximity to a negative charge. To determine if charge repulsion was playing a role in the lack of line broadening for the other 5F-Trp residues, especially that of 5F-Trp 45, the experiment was repeated with Gd:DTPA, which has a net charge of 0. However, as with Gd:EDTA, the only resonance observed to broaden significantly was that of 5F-Trp 158 (Figure 2-4B). The possibility that the effects of Gd:EDTA on 5F-Trp 45 were being obscured by the overlapping 5F-Trp

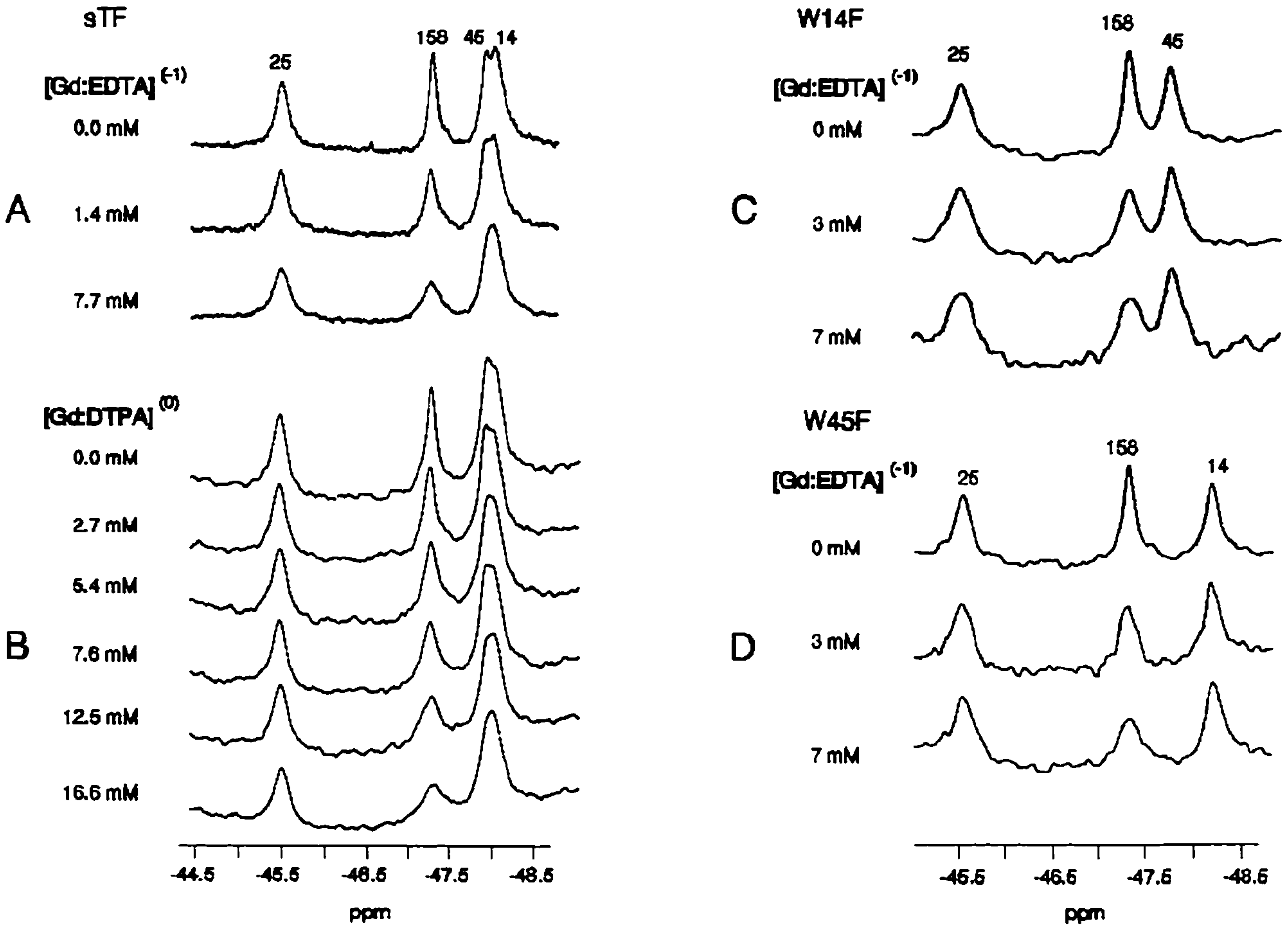


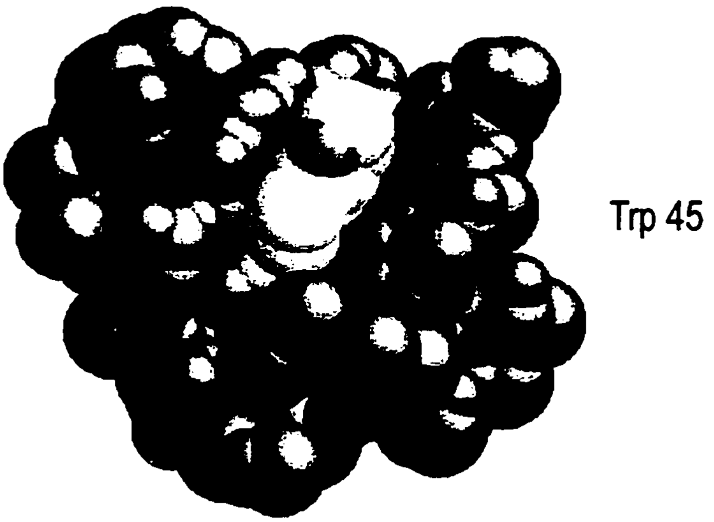
Figure 2-4: Perturbation of $^{19}$F NMR spectra as a function of paramagnetic agent concentration: Titration of wild type sTF (A) with 0-7.7 mM Gd:EDTA, and (B) with 0-16.6 mM Gd:DPTA. Titration of W14F (C) with 0-7.7 mM Gd:EDTA, and (D) with 0-16.6 mM Gd:DPTA.

14 resonance was considered. To observe perturbation of each of these resonances separately, Gd:EDTA titrations were made on the W14F and W45F mutant proteins. As observed for wild type, only the 5F-Trp 158 resonance underwent substantial line broadening (Figures 2-4C and 2-4D), indicating that the fluorine atom on 5F-Trp 45 is not solvent accessible.

In general, the solvent accessibility of the Trp residues, as measured by fluorescence quenching, correlates well with solvent accessibility area of the entire indole ring calculated from the x-ray crystal (Hasselbacher et al., 1995a). The paramagnetic line broadening results, on the other hand, do not show such a correlation. However, as shown in Figure 2-5, position 5 on the indole ring of Trp 45 is completely buried in the matrix of the protein while the corresponding ring position of Trp 158 is fully exposed to solvent. This would account for the difference between the results from fluorescence and absorption versus NMR spectroscopy.

### The utility of combining $^{19}$F NMR spectroscopy with fluorescence spectroscopy

$^{19}$F NMR and fluorescence spectroscopy yield complementary but not identical information on the solvent accessibility of residues in proteins in solution. In particular, $^{19}$F NMR spectroscopy makes observations about one atom on a Trp residue while fluorescence spectroscopy makes observations about the average environment of the entire side chain. Additionally, $^{19}$F NMR spectroscopy is very sensitive to subtle alterations in the local environments of the side chains of the Trp residues that do not affect the overall structure of the protein. This is evident from the observed shifts in the resonance peaks associated with



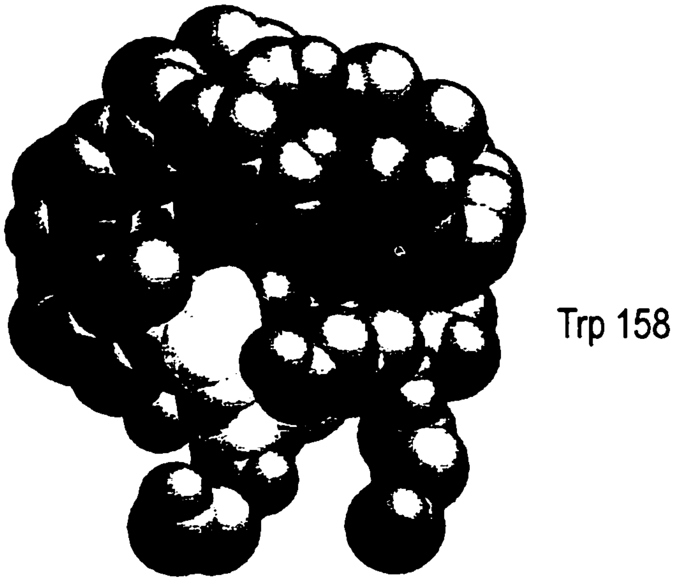


Figure 2-5: Rasmol (Sayle & Milner-White, 1995) space-filling representation of the local environments of Trp 45 and Trp 158 in sTF based on the 1.7 Å resolution x-ray crystal structure by Muller et al. (1996). The protein atoms within 10 Å of the C-5 atom of each indole ring are shown in gray. The indole ring atoms are shown in white except for C-5, which is black, and N-1, which is dark gray. It should be noted that C-5 of Trp 45 is essentially buried in the protein matrix, while C-5 of Trp 158 is essentially exposed to solvent.

Domain I Trp residues in the W14F and W45F mutants. Banner et al. (1996) indicated that the sTF main chain backbone does not experience structural alterations upon binding VIIa. However, this does not presuppose that the side chains do not undergo structural alterations that would facilitate formation of the complex between sTF and VIIa. Thus, it is anticipated that $^{19}$F NMR spectroscopy will be a useful tool for determining conformational changes in the side chains of sTF resulting from formation of the binary complex with VIIa or ternary complexes with VIIa and ligand or inhibitor.

## REFERENCES:

Ashton, A.W., G. Kemball-Cook, D.J.D. Johnson, D.M.A. Martin, D.P. O'Brien, E.G. Tuddenham, and S.J. Perkins. 1995. Factor VIIa and the extracellular domains of human tissue factor form a compact complex: a study by X-ray and neutron solution scattering. *FEBS Lett.* 374:141-146.

Bach, R. 1988. Initiation of coagulation by tissue factor. *CRC Crit. Rev. Biochem.* 23:339-368.

Bach, R., R. Gentry, and Y. Nemerson. 1986. Factor VII binding to tissue factor in reconstituted phospholipid vesicles: induction of cooperativity by phosphatidylserine. *Biochemistry* 25:4007-4020.

Banner. D.W., A. D'Arcy, C. Chene, F.K. Winkler, A. Guha, W.H. Konigsberg, Y. Nemerson, and D. Kirchhofer. 1996. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature* 380:41-46.

Broze, G.J., Jr. and P.W. Majerus. 1980. Purification and properties of human coagulation factor VII. *J. Biol. Chem.* 255:1242-1247.

Danielson, M.A. and J.J. Falke. 1996. Use of 19F NMR to Probe Protein Structure and Conformational Changes. in *Annual Review of Biophysics and Biomolecular Structure.*

R.M. Stroud, W.L. Hubbel, W.K. Olson, and M.P. Sheetz, editors. Annual Reviews, Inc. Palo Alto. 163-195.

Davie, E.W. 1995. Biochemical and molecular aspects of the coagulation cascade. *Thromb. Haemost.* 74:1-6.

Edgington, T.S., N. Mackman, K. Brand, and W. Ruf. 1991. The structural biology of expression and function of tissue factor. *Thromb. Haemost.* 66:67-79.

Fiore, M.M., P.F. Neuenschwander, and J.H. Morrissey. 1994. The biochemical basis for the apparent defect of soluble mutant tissue factor in enhancing the proteolytic activities of factor VIIa. *J. Biol. Chem.* 269:143-149.

Gerig, J.T. 1994. Fluorine NMR of Proteins. *Prog. NMR Spectrosc.* 26:293-370.

Harlos, K., D.M. Martin, D.P. O'Brien, E.Y. Jones, D.I. Stuart, I. Polikarpov, A. Miller, E.G. Tuddenham, and C.W.G. Boys. 1994. Crystal structure of the extracellular region of human Tissue factor [published erratum appears in Nature 1994 Oct 20;371(6499): 720]. *Nature* 370:662-666.

Hasselbacher, C.A., E. Rusinova, E. Waxman, R. Rusinova, R.A. Kohanski, W. Lam, A. Guha, J. Du, T.C. Lin, I. Polikarpov, C.W.G Boys, Y. Nemerson, W.H. Konigsberg, and J.B.A. Ross. 1995a. Environments of the four tryptophans in the extracellular domain of human tissue factor: comparison of results from absorption and fluorescence difference spectra of tryptophan replacement mutants with the crystal structure of the wild-type protein. *Biophys. J.* 69:20-29.

Hasselbacher, C.A., R. Rusinova, E. Rusinova, and J.B.A. Ross. 1995b. Spectral Enhancement of Recombinant Proteins with Tryptophan Analogs: The Soluble Domain of Human Tissue Factor. in *Techniques in Protein Chemistry.* J.W. Crabb, editor. Academic Press, New York. 349-356.

Luck, L. A. and J.J. Falke. 1991. Open Conformation of a Substrate-Binding Cleft: $^{19}$F NMR Studies of Cleft Angle in the D-Galactose Chemosensory Receptor. *Biochemistry* 30:6484-6490.

Miletich, J.P., G.J. Broze,Jr., and P.W. Majerus. 1981. Purification of human coagulation factors II, IX, and X using sulfated dextran beads. *Methods Enzymol.* 80:221-228.

Muller, Y.A., M.H. Ultsch, and A.M. de Vos. 1996. The crystal structure of the extracellular domain of human tissue factor refined to 1.7 Å resolution. *J. Mol. Biol.* 256:144-159.

Muller, Y.A., M.H. Ultsch, R.F. Kelley, and A.M. de Vos. 1994. Structure of the extracellular domain of human tissue factor: location of the factor VIIa binding site. *Biochemistry* 33:10864-10870.

Nemerson, Y. 1988. Tissue factor and hemostasis [published erratum appears in Blood 1988 Apr.;71(4):1178]. *Blood* 71:1-8.

Nemerson, Y. 1995. Tissue factor: then and now. *Thromb. Haemost.* 74:180-184.

Nemerson, Y. and R. Gentry. 1986. An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage [published erratum appears in Biochemistry 1987 Feb 10;26(3):974]. *Biochemistry* 25:4020-4033.

Neuenschwander, P.F. and J.H. Morrissey. 1992. Deletion of the membrane anchoring region of tissue factor abolishes autoactivation of factor VII but not cofactor function. *J. Biol. Chem.* 267:14477-14482.

Østerud, B. 1997. Tissue Factor: a complex biological role. *Thromb. Haemost.* 78:755-758.

Ross, J.B.A., A.G. Szabo, and C.W.V. Hogue. 1997. Enhancement of Protein Spectra with Tryptophan Analogs: Fluorescence Spectroscopy of Protein-Protein and Protein-Nucleic Acid Interactions. *Methods Enzymol.* 278:151-190.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Press, New York.

Sayle, R.A., and E.J. Milner-White. 1995. RasMol: Biomolecular graphics for all. *Trends in Biochemical Sciences*. 20:374-376.

Snavely, M.D., J.B. Florer, C.G. Miller, and M.E. Maguire. 1989. Magnesium transport in *Salmonella typhimurium*: expression of cloned genes for three distinct $Mg^{2+}$ transport systems. *J. Bacteriol.* 171:4752-4760.

Sörensen, B.B., E. Persson, P.-O. Freskgard, M. Kjalke, M. Ezban, T. Williams, and L.V.M. Rao. 1997. Incorporation of an active site inhibitor in factor VIIa alters the affinity for Tissue Factor. *J. Biol. Chem.* 272:11863-11868.

Waxman, E. 1993. Thermodynamic and hydrodynamic behavior of the Tissue Factor:Factor VII(a) complex. Dissertation, Mount Sinai School of Medicine of the City University of New York.

Waxman, E., W.R. Laws, T.M. Laue, Y. Nemerson, and J.B.A. Ross. 1993a. Human factor VIIa and its complex with soluble tissue factor: evaluation of asymmetry and conformational dynamics by ultracentrifugation and fluorescence anisotropy decay methods. *Biochemistry* 32:3005-3012.

Waxman, E., J.B.A. Ross, T.M. Laue, A. Guha, S.V. Thiruvikraman, T.C. Lin, W.H. Konigsberg, and Y. Nemerson. 1992. Tissue factor and its extracellular soluble domain:

the relationship between intermolecular association with factor VIIa and enzymatic activity of the complex. *Biochemistry* 31:3998-4003.

Waxman, E., E. Rusinova, C.A. Hasselbacher, G.P. Schwartz, W.R. Laws, and J.B.A.Ross.1993b. Determination of the tryptophan:tyrosine ratio in proteins. *Anal. Biochem.* 210:425-428.

# CHAPTER 3

## Spectral Enhancement of Proteins with 5-Fluorotryptophan: The Fluorescence Properties of 5-Fluorotryptophan in Soluble Human Tissue Factor

From Zemsky, et al., manuscript submitted

## INTRODUCTION

Fluorescence spectroscopy is a useful tool for probing protein structure and conformational dynamics. In particular, steady-state or time resolved fluorescence spectroscopy can be used to monitor the local environment of probes, binding events, alterations in structure, and to predict size and hydrodynamic shape of proteins (reviewed by Eftink, 1991). Both extrinsic and intrinsic probes are used for fluorescence studies. Advantages of extrinsic probes, such as fluorescein or rhodamine derivatives, include high quantum yields and signals that are easily separable from the native fluorescence of proteins and nucleic acids. However, extrinsic probes suffer from limitations. First, it is not always possible to place the probe at a specific site. Secondly, introduction of the probe may affect protein function adversely due to steric or other chemical alterations. As an alternative, the intrinsic fluorescence of proteins may be used to probe protein structure or function. The intrinsic fluorescence of proteins is a result of the tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) residues that are present. Trp has the strongest absorption and can be selectively excited at wavelengths longer than 295 nm. Protein fluorescence is dominated by Trp fluorescence because at shorter wavelengths, where all three aromatic residues are excited, much of the excitation energy is transferred to the Trp residues via resonance energy transfer (Eftink, 1991).

Most proteins contain one or more Trp residues making them amenable to investigation by intrinsic Trp fluorescence. However, intrinsic fluorescence studies of protein-protein or protein-DNA systems are limited. In general, it is impossible to study the behavior of just one member of a protein complex because the closely similar absorbance spectra of the different proteins do not allow selective excitation of the Trp residues in just one of the proteins. Similarly, the wavelength range of DNA absorbance is equivalent to that of Trp, and the high extinction of DNA completely overwhelms that of Trp, making it impossible to selectively excite Trp in the presence of DNA.

One approach to observing changes in Trp fluorescence in a protein in the presence of another protein or DNA is substitution of the Trp residues with analogs of Trp that have altered absorbance and fluorescence characteristics (Figure 3-1), thereby generating spectrally enhanced proteins (SEPs) (Ross et al., 1997). Nearly thirty years ago, Schlesinger (1968) noted the altered absorbance and fluorescence characteristics of alkaline phosphatase when azatryptophan or tryptazan were incorporated during protein expression using removal of inorganic phosphate to activate synthesis of the protein. Schlesinger did not note the potential utility of these analogs as spectroscopic tools, and for many years Trp analogs had little if any impact on methods for studying protein structure and function. One reason is due to the toxicity of the analogs to the cells used to produce protein (Pratt & Ho, 1975; Barlati & Ciferri, 1970; Pardee et al., 1956; Lark, 1969). However, the advances in recombinant DNA methodology have provided a means to synthesize analog-containing proteins under strongly regulated promoters, which help moderate the toxicity of the analogs. Many groups have been able to incorporate Trp analogs into recombinant proteins with efficiencies of 90% or greater (*c.f.*, Ross et al., 1997). Moreover, many of the analogs containing proteins generated have activities and stability similar to those of the original proteins (Wong & Eftink, 1997; Ross et al., 1992; Soumillion et al., 1995; Laue et al., 1993; Hogue et al., 1992).

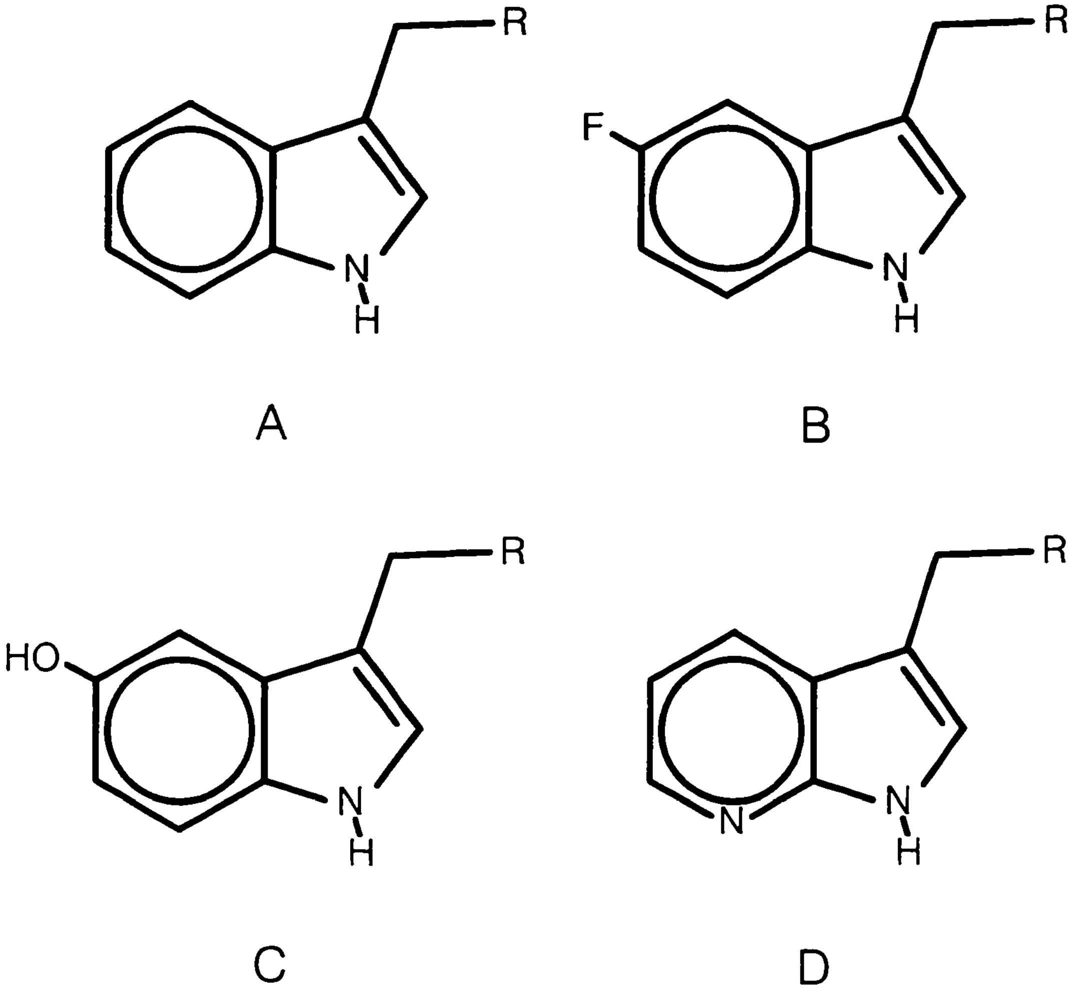


Figure 3-1: Tryptophan analogs. A. Tryptophan, B. 5-Fluoro-Tryptohan, C. 5-Hydroxy-Tryptophan, D. 7-Aza-Tryptophan

Recent fluorescence studies have demonstrated the feasibility of using Trp analogs to study a single protein within a protein complex by selective excitation of Trp analogs in SEPs that are in complexes with other Trp-containing proteins. One example is the steady-state and time-resolved fluorescence studies of protein complexes composed of VP16 protein containing either 5-hydroxy-Trp (5OH-Trp) or 7-aza-Trp (7A-Trp) bound to either the TATA-box binding protein (TBP) or transcription factor B (TFIIB) (Shen , 1996).

Trp fluorescence has been of interest as a tool to study the interaction between human Tissue Factor (TF) and the enzyme Factor VIIa (VIIa). TF is a membrane-bound protein that upon tissue damage triggers the extrinsic pathway of blood coagulation by binding and subsequently activating VIIa (Østerud, 1997; Kirchhofer & Nemerson, 1996; Edgington et al., 1997). In the absence of TF, VIIa has virtually no proteolytic activity towards either peptide or macromolecular substrates (Ruf et al., 1991; Ruf & Edgington, 1994; Bom & Bertina, 1990). It is desirable to understand how the activation of VIIa proteolytic activity results from binding to TF (Nemerson & Gentry, 1986). Previously, Hasselbacher et al. in the lab had developed a method of difference spectroscopy to determine the individual absorbance and fluorescence spectra for each Trp residue in soluble TF (sTF), which is a soluble, functional truncation mutant of the extracellular domain of TF (Hasselbacher et al., 1995a). The next step was to apply difference absorption and fluorescence spectroscopy to investigate the effects on the local environments of Trp residues of sTF that might result from association with VIIa. However, to study the complex with VIIa, it was necessary to generate recombinant sTF in a form that could be excited selectively. To this end, incorporation of both 5OH-Trp and 7A-Trp into sTF was attempted. Unfortunately, incorporation of these analogs was inefficient and most of the expressed protein was insoluble (Hasselbacher et al., 1995b). Then the possibility of using 5-fluoro-Trp (5F-Trp), which also has a red-shifted absorption compared to Trp, was investigated to generate spectrally enhanced sTF. F-Trp analogs along with fluoro-Tyr and fluoro-Phe analogs have been used extensively in $^{19}$F NMR spectroscopic studies (Danielson & Falke, 1996). However, these fluorinated amino acid analogs generally have not been used for fluorescence studies. The absorption shifts exhibited by F-Trp analogs are significantly smaller than those of 5OH-Trp or 7A-Trp. As a result, F-Trp analogs generally would be considered less desirable for generating SEPs (Ross et al., 1992). For example, the absorbance spectrum of 6F-Trp is completely overlapped by that of Trp, making it impossible to excite selectively, and 4F-Trp has a blue-shifted absorbance spectrum compared to that of Trp. 4F-Trp could be potentially useful for studying protein-protein complexes; it does not fluoresce and thus can be used to generate a non-fluorescent protein (Bronskill & Wong, 1988).

In addition to its red-shifted absorbance, which makes 5F-Trp potentially useful for study of protein-protein or protein-nucleic acid interactions, the fluorine atom in 5F-Trp makes the analog potentially useful for complementary $^{19}$F NMR studies. Recently the biological activity and $^{19}$F NMR spectral characteristics of 5F-Trp sTF were reported (Zemsky et al., 1998). It was found that 5F-Trp had no effect of sTF function, and the $^{19}$F NMR results about the local Trp environments were compared with those obtained from the difference absorption and fluorescence of the Trp residues. In contrast with fluorescence spectroscopy, which generates information on the local environments surrounding the entire aromatic ring of the Trp residue, $^{19}$F NMR generates information on the local environment of the atom attached to the 5 carbon of the indole ring. For example, the fluorescence of Trp 45 indicates that its indole ring is partially solvent exposed, but, the $^{19}$F NMR data revealed that position 5 of this ring is shielded from solvent and buried in the protein matrix. Moreover, although the single Trp-to-Phe (or Tyr) replacement mutants used to make $^{19}$F resonance assignments are fully functional as cofactors for VIIa, the $^{19}$F NMR spectra for the remaining 5F-Trp residues suggested that the single Trp-to-Phe (or Tyr) mutations result in subtle structural perturbations. Here, the absorbance and fluorescence spectra of 5F-Trp are compared with that of Trp in sTF and the single Trp replacement mutants to assess the utility of 5F-Trp as an analog for spectral enhancement of proteins.

## MATERIALS AND METHODS

### Reagents

*Materials.* 5-d,l-Fluorotryptophan was obtained from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI) and Acros (Pittsburgh, PA). Recombinant human factor VIIa was a gift from NOVO Nordisk (Denmark), and factor X was purified from human plasma by the methods of Miletich et al. (1981) and Broze and Majerus (1980). 1,2-Dioleoyl-*sn*-glycero-3-phosphotidylserine and 1,2-dioleoyl-*sn*-glycero-3-phophotidylcholine were obtained from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate Spectrozyme was obtained from American Diagnostica Inc. (Greenwich, CT).

Guanidinium chloride (GdmCl) was purchased from Heico Chemicals (Delaware Water Gap, PA).

### Protein Expression

sTF and single Trp replacement mutants of sTF (Trp to Phe or Trp to Tyr) were expressed in *Escherichia coli* Trp auxotroph CY15077 EA2 (Ross et al., 1997; Hasselbacher et al., 1995b). Expression and purification conditions are identical to those reported in Zemsky et al. (1998).

### Chromogenic Assay for sTF Activity as a Cofactor for VIIa

The chromogenic assay to monitor the cofactor activity of wild type sTF and the mutant proteins with and without 5F-Trp was performed as previously described (Hasselbacher et al., 1995a). As reported by Zemsky et al. (1998), 5F-Trp has no effect on the cofactor activity.

### Ultraviolet Absorbance Spectroscopy

Absorption spectra were measured at room temperature with a dual-beam Hitachi U-3210 spectrophotometer. Protein sample buffer was composed of 0.05 M Tris, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 1 mM $NaN_3$, unless otherwise indicated. Spectra were obtained for both native and denatured (6 M GdmCl) samples of each protein between 260 and 340 nm. The absorbance spectra of the denatured proteins were used to calculate the percent incorporation of 5F-Trp into each protein by LINCS analysis as previously described

(Zemsky et al., 1998; Waxman et al., 1993). As described below, incorporation of 5F-Trp was less than 100% efficient in the mutant proteins, and it was necessary to account for residual Trp contributions in the 5F-Trp protein absorption and fluorescence emission spectra.

### Fluorescence Spectroscopy

Steady-state fluorescence emission spectra (8 nm excitation and emission band-passes) were obtained with a SLM 4800 spectrofluorometer, modified in our laboratory for single-photon counting, using magic angle polarization to avoid intensity artifacts resulting from molecular rotation. All samples were measured at 20°C. Protein emission spectra were measured over the wavelength region between 300 and 500 nm using 295-nm excitation to avoid excitation of Tyr fluorescence. At 295 nm, however, there is excitation of both Trp and 5F-Trp. This wavelength was used to permit direct comparison of the fluorescence yields of proteins containing either Trp or 5F-Trp, which is straightforward providing there is complete substitution of Trp by 5F-Trp. Spectra were corrected for the wavelength response of the fluorometer.

The relative quantum yields of 5F-Trp and Trp were determined using excitation at 280 nm which is approximately the maximum of their $S_1 \leftarrow S_0$ bands, from their integrated emission between 285 and 500 nm. Excitation at 280 nm was used to minimize errors in excitation bandwidth that result from environment-dependent differences in spectral shape, and the absorbance of each sample was 0.1 (1 cm path length) using neutral pH aqueous buffer or dioxane as solvent.

## Correction of 5F-Trp Protein Spectra for Incomplete Trp Replacement

The efficiency of Trp residue replacement by Trp analogs is variable and must be taken into account for interpretation of the absorption and fluorescence spectra. According to analysis either by LINCS or mass spectrometry, the Trp sites in wild type sTF are completely replaced by 5F-Trp. For the mutant proteins, however, only 70 to 80% replacement was achieved (Zemsky et al., 1998). Based upon $^{19}F$ NMR spectroscopy of the 5F-Trp wild type and mutant proteins, 5F-Trp was incorporated randomly into the Trp sites in each protein (Zemsky et al., 1998). As a result, there is no bias in the potential contribution of each Trp site to the protein "5F-Trp" spectrum. This makes subtraction of the Trp contribution to the fluorescence of a 5F-Trp containing protein feasible.

In order to compare the fluorescence emission spectra with each other and to generate difference spectra it was necessary to normalize the spectra with each other. It was not possible to use concentration as calculated by absorbance at 280 nm to normalize the spectra because the relative contributions from Trp and 5F-Trp varied between proteins. As a result, it was first necessary to normalize the concentrations of the different proteins and then to eliminate the Trp contributions to each spectrum. The correction for Trp contribution in a 5F-Trp containing protein was made using four assumptions. First, in a denatured protein each 5F-Trp residue contributes equally to the emission. Second, each Trp residue contributes equally to the emission. The first two assumptions are based upon the assumption that GdmCl denatures the protein completely and each 5F-Trp or Trp residue is exposed to an equivalent environment. This does not take into account interactions with neighboring amino acids. Third, in the denatured polypeptide chain, the relative contributions of Trp and 5F-Trp to the total emission are dependent on their relative extinction at the wavelength of excitation, their relative quantum yields, and the ratio of Trp and 5F-Trp residues. Fourth, Trp is completely replaced by 5F-Trp in wild type sTF. The last assumption is supported by the results from LINCS analysis and mass spectra (Zemsky et al., 1998).

Based upon the above assumptions, the denatured proteins were used to normalize protein concentrations to each other. The intensities of the integrated emission spectra of denatured 5F-Trp sTF and denatured Trp sTF were normalized to each other using concentration as calculated from absorbance at 280 nm. These normalized spectra were assumed to provide the relative fluorescence yields for all 4 5F-Trp ($\varphi_{5F\text{-}Trp.\ GdmCl}$) in denatured 5F-Trp sTF and for all 4 Trp ($\varphi_{Trp,\ GdmCl}$) in denatured Trp sTF, respectively.

The emission spectrum of a single-Trp replacement mutant of sTF expressed in the presence of 5F-Trp should have 75% of the intensity of the 5F-Trp sTF emission spectrum if 5F-Trp replacement is complete. Since 5F-Trp replacement was not complete, the emission spectra of the mutants was a result of both 5F-Trp and Trp contributions. Theoretical yields were calculated for each mutant taking into account the percent 5F-Trp and percent Trp residues contributing to each sample. For a given protein with n Trp residues and a fractional incorporation of x, the calculated fluorescence yield is

$$\varphi_{\mathrm{mutant}} = (n) \times [(x) \times (\varphi_{\mathrm{5F\text{-}Trp,\,GdmCl}}) + (1\text{-}x) \times (\varphi_{\mathrm{Trp,\,GdmCl}})] \tag{1}$$

The theoretical yields were then compared to the measured emission intensities of the denatured mutant protein spectra. The ratio of the calculated yield to the measured yield was used to scale the intensities of the spectra of the proteins in the native state.

Correction of a 5F-Trp spectrum of a native mutant for the Trp contribution was calculated by subtracting the Trp contribution using the relative yields of each single-Trp replacement mutant previously determined by Hasselbacher et al. (1995a). First, the Trp fluorescence spectrum of the mutant was scaled to reflect the percent incorporation of 5F-Trp. Then the scaled Trp spectrum was subtracted from the 5F-Trp mutant spectrum and the resultant spectrum, $F\,(\mathrm{mutant})\,d\lambda$, was re-scaled to represent 100% incorporation of 5F-Trp. For a given protein

$$F\,(\mathrm{5F\text{-}Trp\ mutant_{corrected}})\,d\lambda = 1/x \times [F\,(\mathrm{5F\text{-}Trp\ mutant})\,d\lambda - (1\text{-}x) \times F\,(\mathrm{mutant})\,d\lambda] \tag{2}$$

For the W25Y and W158F mutants, the relevant Trp emission spectrum to subtract was the wild type because Trp-25 and Trp-158 are "silent" in the Trp-containing protein (Hasselbacher et al., 1995a).

Following the approach of Hasselbacher et al. (1995a), difference spectra were generated for each 5F-Trp residue by subtracting the corrected mutant 5F-Trp spectrum from that of 5F-Trp sTF. This difference spectrum putatively represents the fluorescence of 5F-Trp at the site of mutation.

# RESULTS AND DISCUSSION

## Absorbance Characteristics of 5F-Trp in Proteins

The absorbance of 5F-Trp relative to Trp and Tyr needed to be determined for quantitative analysis of 5F-Trp incorporation by LINCS analysis. To this end, the absorbance of the amino acids 5F-Trp and Trp were measured in TBS buffer with 6 M GdmCl from 260 to 340 nm. The extinction of 5F-Trp relative to Trp and Tyr in a polypeptide chain was established by comparing the LINCS analysis of sTF containing Trp with that containing 5F-Trp, both denatured in 6M GdmCl. First, it was determined that the absorbance spectrum of 5F-Trp containing sTF could be fit satisfactorily with the absorbance spectra for 5F-Trp and N-acetyl-tyrosinamide (NATyrA). This suggested that 5F-Trp could replace with close to 100% efficiency the Trp residues in wild type sTF, which was confirmed by the mass spectroscopy. Knowledge of the number of Tyr residues and Trp sites in sTF (eleven and four, respectively) allowed calculation of the molar extinction of "typical" 5F-Trp residues in a GdmCl denatured polypeptide chain. Accordingly, an $\varepsilon_{280}$ value of 6,150 $cm^{-1}$ $M^{-1}$ was obtained for 5F-Trp when using an $\varepsilon_{280}$ value of 1010 $cm^{-1}$ $M^{-1}$ for NATyrA. The latter value is based on an $\varepsilon_{280}$ value of 5,480 $cm^{-1}$ $M^{-1}$ for N-acetyl-tryptophanamide (NATrpA), and was determined by Waxman et al. (1993) from the absorption spectra of a representative series of soluble proteins and peptides in 6M GdmCl and their Trp-to-Tyr ratio. The absorbance of 5F-Trp is clearly greater than that of Trp over the entire wavelength range above 260 nm (Figure 3-2), and

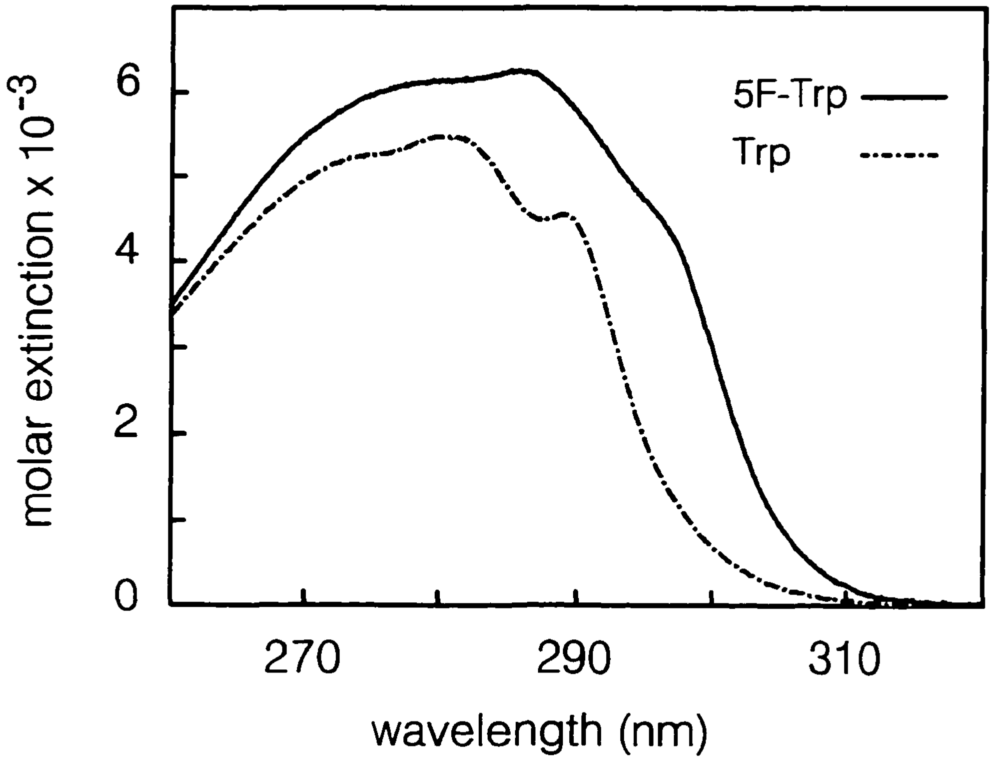


Figure 3-2: Absorption spectra of 5F-Trp and Trp (in 0.05 M Tris, pH 7.4, 0.1 M NaCl, 0.001 M EDTA, 0.001 M $NaN_3$ and 6 M GdmCl).

in particular the difference in extinction from 300 to 310 nm should be noted. Similarly, the absorbance spectrum of 5F-Trp sTF has greater extinction than sTF over the entire range from 260 to 340 nm (Figure3-3). Furthermore, 5F-Trp displays the same extended absorption range from 300 to 310 nm. It is this difference that is expected to permit selective excitation of 5F-Trp in the presence of Trp, in a manner similar to the analogs 5OH-Trp and 7A-Trp (Ross et al., 1997).

### Fluorescence Characteristics of 5F-Trp versus Trp

The fluorescence emission and relative quantum yield of 5F-Trp are similar to those of Trp, with essentially identical responses to changes in the local environment. The relative quantum yields in each buffer were calculated by integrating the non-normalized spectra and directly comparing the yields. Figure 3-4 shows, for example, the fluorescence spectra obtained using aqueous buffer and dioxane, a low dielectric solvent. In both solvents, the 5F-Trp spectra is red-shifted approximately 4 nm relative to those of Trp. The similarities between the Trp and 5F-Trp fluorescence emission spectra suggest that for steady-state fluorescence emission studies, both amino acids will report similar information about the local environment.

### Fluorescence Emission Spectra of 5F-Trp sTF and its Single Trp Replacement Mutants

5F-Trp incorporation into sTF and single Trp replacement mutants of sTF was determined using LINCS analysis and mass spectrometry. As described in Zemsky et al., (1998), the wild type protein was found to have essentially complete replacement of Trp

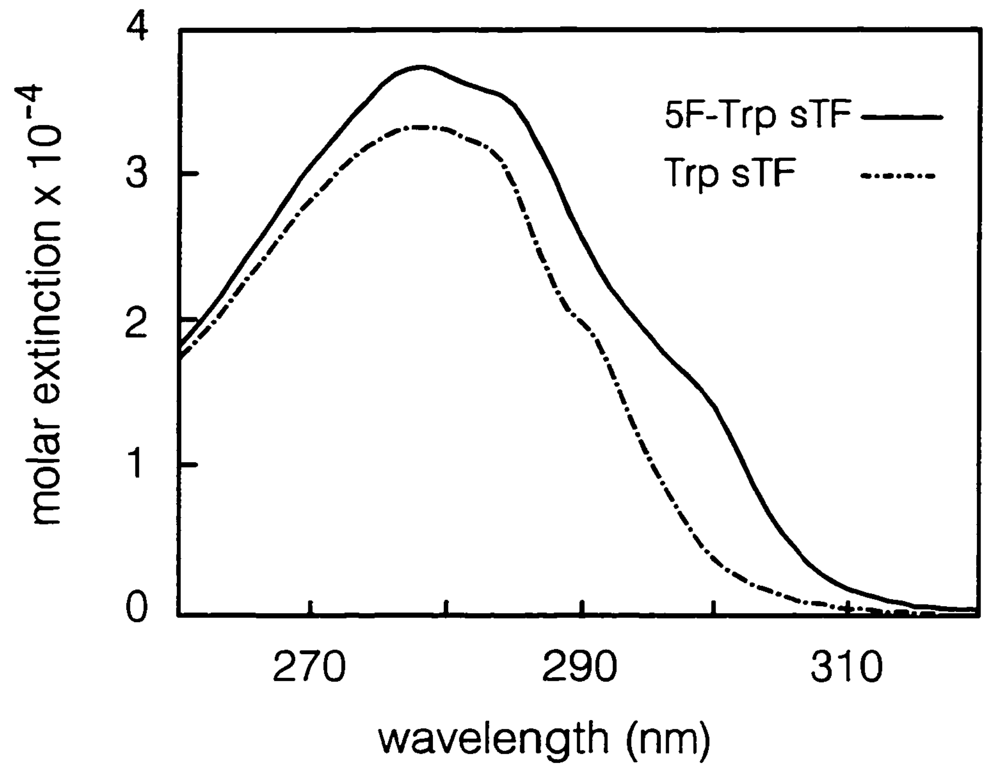


Figure 3-3: Absorption spectra of native 5F-Trp sTF and Trp sTF (in 0.05 M Tris, pH 7.4, 0.1 M NaCl, 0.001 M EDTA, and 0.001 M $NaN_3$).

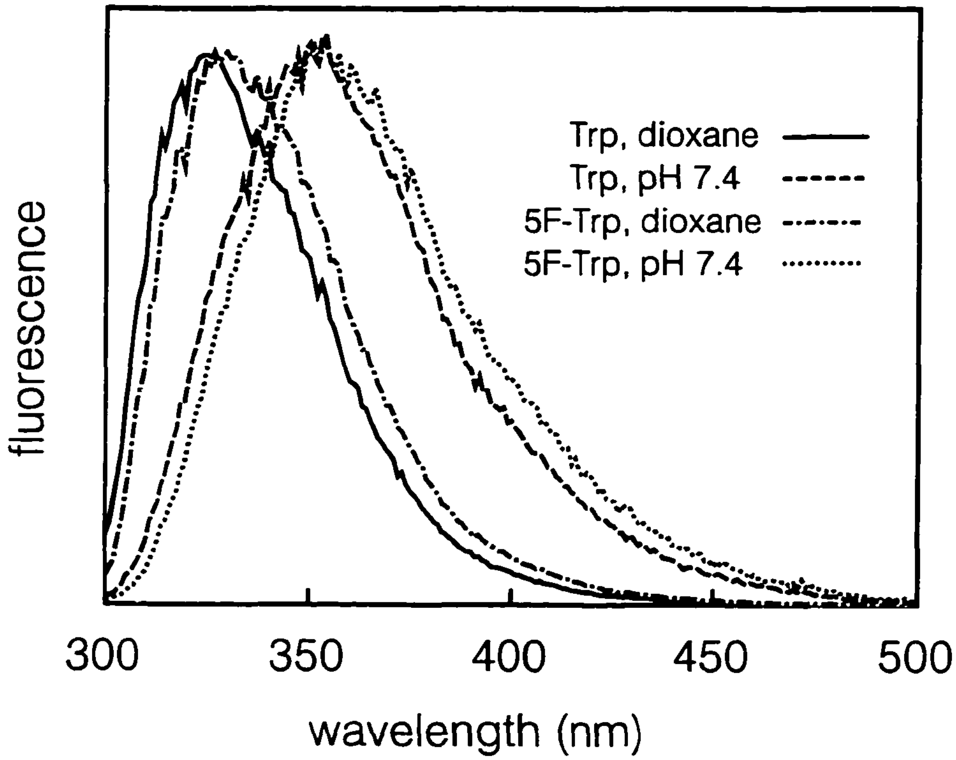


Figure 3-4: Fluorescence emission spectra of Trp and 5F-Trp in either pH 7.4 buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, 0.001 M $NaN_3$) or dioxane. Samples were excited at 280 nm. Spectra were corrected for the wavelength response of the fluorometer and peak normalized.

by 5F-Trp according to both analytical methods. Incorporation of 5F-Trp into the mutant proteins was lower than in the wild type, yielding protein samples with 77 to 83% of the Trp residues replaced by 5F-Trp. As a result, the mutant spectra needed to be corrected for Trp contribution to absorption and fluorescence (see **Methods**). The 5F-Trp mutant fluorescence spectra, corrected for Trp contribution and for the wavelength response of the fluorometer, are displayed relative to that of sTF in Figure 3-5. Three of the mutant spectrum is less intense than the wild type spectrum, indicating that three of four Trp sites contribute to the 5F-Trp fluorescence. These results are in contrast to those obtained by Hasselbacher et al., (1995) for Trp fluorescence in sTF showing that only the Trp residues at positions 14 and 45 exhibited significant fluorescence. Moreover, the sum of the difference fluorescence spectra representing Trp-14 and Trp-45, which were generated by subtracting the mutant emission spectrum from that of the wild type protein, was equivalent to that of wild type sTF. The other two Trp residues at positions 25 and 158 are in close proximity to disulfide bonds, which have been shown to quench Trp fluorescence in other systems (Longworth, 1971), and are speculated also to do so in Trp sTF. Thus, it was possible to account entirely for the wild type protein fluorescence by the spectra of the single-Trp replacement mutants.

The fluorescence spectroscopy of 5F-Trp in sTF and the single-Trp replacement mutants is clearly more complex than that of Trp. First, nearby disulfide bonds do not appear to quench the fluorescence of 5F-Trp residues as effectively. Either 5F-indole and indole interact differently with disulfide bonds or, alternatively, its neighboring disulfide bond does not cause the quenching of Trp at position 25. Regardless of the mechanism

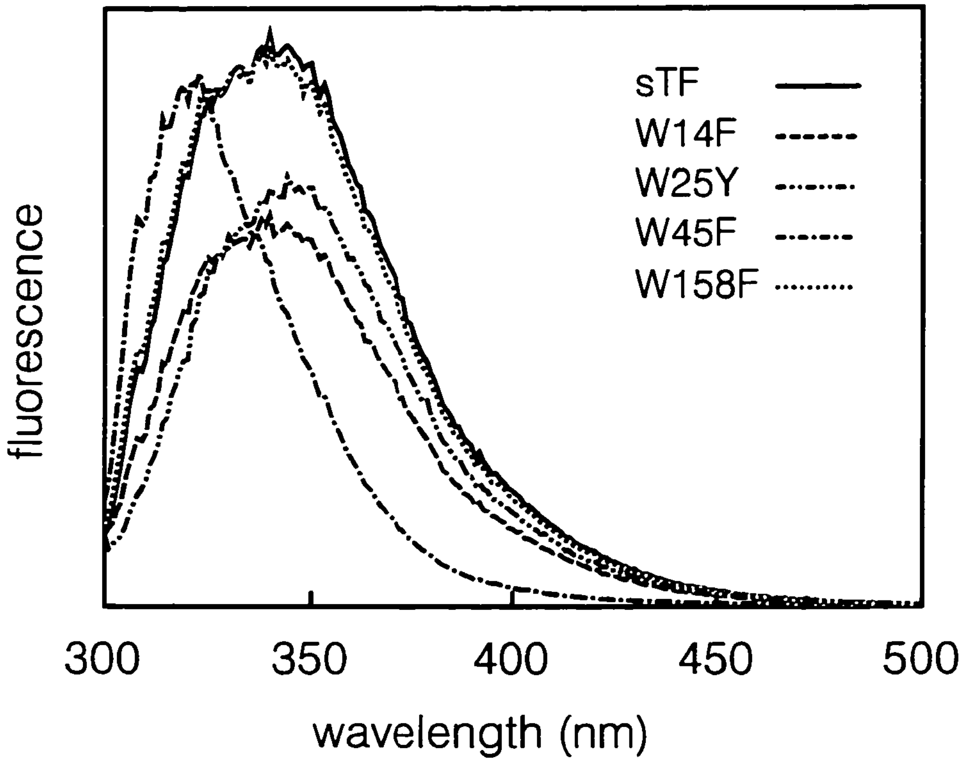


Figure 3-5: Fluorescence emission spectra of sTF and single-Trp-replacement mutants of sTF in pH 7.4 buffer (0.05 M Tris, 0.1 M NaCl, .001 M EDTA, .001 M $NaN_3$). Samples were excited at 295 nm in order to avoid excitation of Tyr residues. Spectra were corrected for the wavelength response of the fluorometer and also for the Trp contamination as described in the **Materials and Methods**.

of the efficient quenching for Trp at position 25, when 5F-Trp occupies this site it is not efficiently quenched and its contribution seems to be similar to that of 5F-Trp at site 14. On the other hand, when site 158 is occupied with Trp there is no detectable fluorescence and when occupied by 5F-Trp, the fluorescence is extremely weak. The second interesting feature of 5F-Trp fluorescence is observed in the spectrum of mutant W45F, which does not fall within the envelope of the wild type spectrum, but is shifted to the blue of the other spectra. It was initially thought that the blue edge fluorescence of W45F was a contaminant. However, three separate protein purifications from three separate preparations displayed the identical, intense blue-shifted fluorescence, which disappeared upon denaturation in GdmCl. This suggested instead that the mutation at site 45, which eliminates the 5F-Trp residue, is responsible for the appearance of the fluorescence at the blue edge of the W45F spectrum. In principle, the W45F mutant spectrum should represent fluorescence from sites 14, 25 and 158. Since site 158 contributes relatively little fluorescence, we expect that the 5F-Trp emission of W45F is dominated by fluorescence from sites 14 and 25. Based on the model spectra (Figure 3-4), 5F-Trp at sites 14 and 25, which are well buried in the protein matrix, would be expected to exhibit blue-shifted emission spectra compared to 5F-Trp at sites 45 and 158, which are relatively more exposed to solvent. Therefore, the blue shift of the W45F fluorescence relative to the wild type spectrum suggests the fluorescence from either or both sites 14 and 25 becomes quenched in the other mutant and wild type proteins by the 5F-Trp residue at position 45.

### Difference Fluorescence Spectroscopy and Non-Additivity of 5F-Trp Spectra

The difference fluorescence spectra for the four single-Trp replacement mutants versus wild type sTF are shown in Figure 3-6. The blue shift of the W45F spectrum compared to that of the wild type protein results in a highly red-shifted difference spectrum with a negative dip at the blue edge. Addition of the individual 5F-Trp difference spectra yields a significantly red-shifted spectrum compared to the wild type fluorescence (Figure 3-7), but with approximately equivalent fluorescent yield as calculated by integration of the spectra. The red shift and negative dip at the blue edge of the W45F difference spectrum accounts for the red shift of the summed difference spectra.

Site 158, which is located more than 40 Å from sites 14, 25, and 45 (see Hasselbacher et al., 1995a), contributes relatively little to the protein emission. On the other hand, 5F-Trp residues at sites 14 and 25, which appear to contribute equally to the fluorescence, are located within 15 Å of site 45. Considering both distances and relative energies of excited states, we considered the possibility that resonance energy transfer (RET) to 5F-Trp at site 45 could account for quenching of 5F-Trp at either or both sites 14 and 25.

### Energy Transfer between 5F-Trp Residues

According to Förster's theory (1948), when donor – acceptor distances are greater than 10 Å, RET is dominated by dipole-dipole coupling, and the transfer rate varies as the inverse sixth power of the distance separating the centers of the absorption and emission

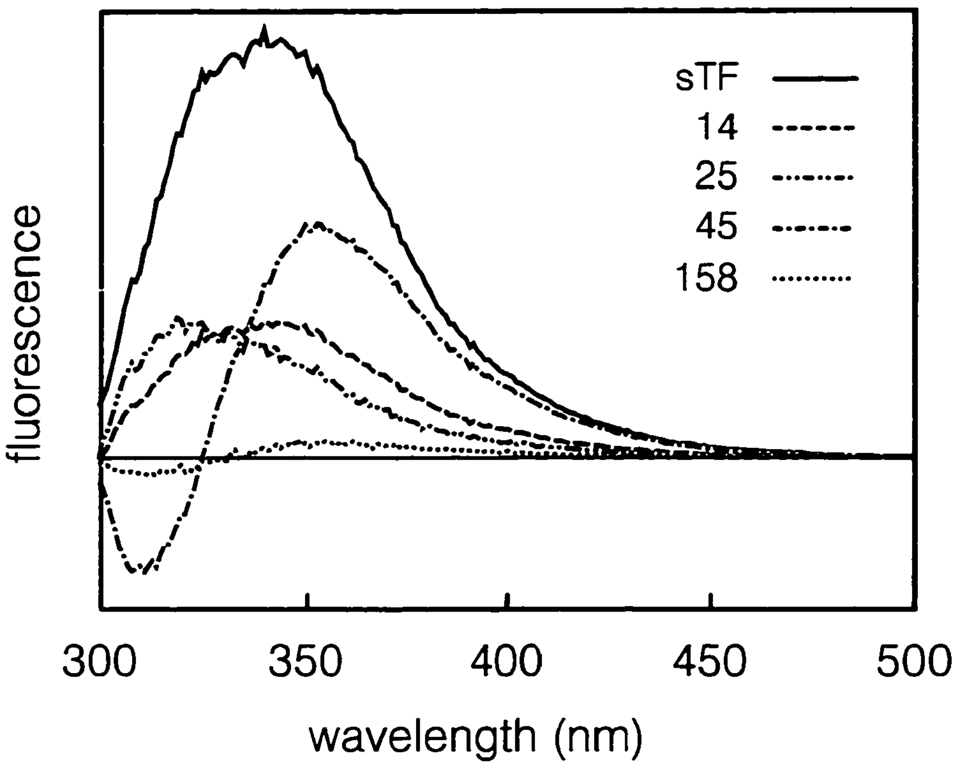


Figure 3-6: Fluorescence emission spectrum of sTF and the difference spectra derived from each mutant spectrum. The difference spectra were calculated as described in **Materials and Methods.**

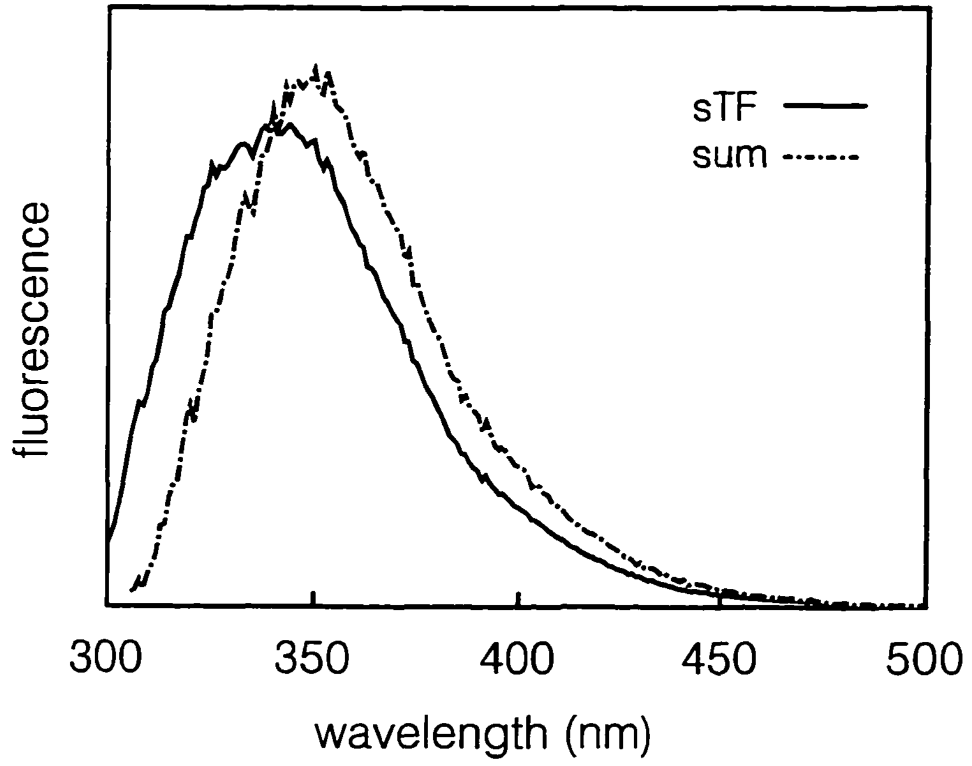


Figure 3-7: Fluorescence emission spectrum of sTF compared with a spectrum obtained by summation of the difference spectra.

transition dipole moments. Based upon the crystal structures of sTF (Muller et al., 1994; Muller et al., 1996; Harlos et al., 1994), Trp sites 14, 25 and 45 are all within 15 Å of each other, sufficiently close to each other to participate in energy transfer. Trp site 158, which is located about 50 Å from the other sites, is much less likely to be involved in RET.

RET also depends the matching between the donor and acceptor energy levels, measured by the extent of donor emission and acceptor absorption spectral overlap,

$$J_{D \rightarrow A} = \int F_D(\lambda)\, \varepsilon_A(\lambda)\, \lambda^4\, d\lambda \qquad (3)$$

where $F_D$ is the donor emission spectrum, its integrated intensity normalized to unity, $\varepsilon_A$ is the acceptor absorbance spectrum in terms of the molar extinction coefficient, and $\lambda$ is the wavelength in nm. The red shift in the 5F-Trp absorbance spectrum, compared with that of Trp, is larger than the red shift in the fluorescence. This decreases slightly the Stoke's shift between absorption and fluorescence, thereby increasing the spectral overlap and the possibility of energy transfer. Table 2 lists overlap integrals calculated for Trp and 5F-Trp in model solvents. Calculations were made using the absorbance spectrum of either Trp or 5F-Trp in 6M GdmCl as an acceptor based upon the observation that this spectrum is very similar to the difference absorption spectrum of Trp 45. The donor fluorescence emission spectra of Trp or 5F-Trp in either dioxane or TBS, which model non-polar and polar environments respectively, were used for calculations.

Table 2. Overlap Integral and $R_o$ Values for 5F-Trp and Trp Donor-Acceptor Pairs.

| Donor | Acceptor | J ( $M^{-1}cm^{6}$ ) | $R_o$ (Å) |
|---|---|---|---|
| 5F-Trp14 + 5F-Trp25 | 5F-Trp 45 | $1.66 \times 10^{-16}$ | 11.9 |
| 5F-Trp, dioxane | 5F-Trp, 6M GdmCl | $3.79 \times 10^{-17}$ | 9.3 |
| Trp, dioxane | Trp, 6M GdmCl | $2.64 \times 10^{-17}$ | 8.8 |
| 5F-Trp, TBS | 5F-Trp, 6M GdmCl | $3.85 \times 10^{-18}$ | 6.4 |
| Trp, TBS | Trp, 6M GdmCl | $2.99 \times 10^{-18}$ | 6.1 |

The overlap integral between the acceptor and donor pair, $J_{AD} = \int F_D(\lambda)\ \varepsilon_A(\lambda)\lambda^4\ d\lambda$, where $F_D$ is the spectral distribution of the fluorescence of the donor, normalized to unity, $\varepsilon_A$ is the molar decadic absorption coefficient of the acceptor and $\lambda$ is the wavelength (Schiller, 1972). $R_O$, the distance at which energy transfer is 50% efficient, is calculated based upon Eisinger et al. (1969) where $R_o^6 = (8.8 \times 10^{25})\Phi_D\kappa^2 n^{-4} J_{AD}$. $\Phi_D$ is the quantum yield and was assigned a value of 0.1 (Schiller, 1972). $\kappa^2 n^{-4}$ is assigned a value of 0.2 based upon $\kappa^2$ having a value of 2/3 and water having a refractive index of 0.74 (Schiller, 1972).

The third factor which RET depends upon is the relative orientation between donor and acceptor. The role of the orientation factor, $\kappa^2$, was also considered for RET to site 45. Although $\kappa^2$ may vary from 0 to 4, a value of 2/3 is often used, accompanied by the explanation that this value strictly applies only when the donor and acceptor dipoles are isotropically averaged on a time scale that is much faster than the fluorescence lifetime of the donor. It is rare that the conditions for which $\kappa^2 = 2/3$ are satisfied, and its value will depend also on the possibility of dipole-dipole interaction with more than one acceptor transition dipole. The two lowest excited electronic states of indole and 5-methoxy indole are the $^1L_a$ and $^1L_b$ states, and information regarding the directions of their respective transition moments within molecular coordinates are available from theoretical calculations and experimental data (see review by Callis, 1997). Thus, in principle it is possible to calculate $\kappa^2$ using protein crystal structure coordinates. This calculation is problematic, however, because the two absorption transitions overlap significantly with the blue side of the fluorescence, and the extent of their overlap also depends upon local environment. Furthermore, depending upon the local environment, either $^1L_a$ or $^1L_b$ could be the lower lying excited state, and therefore one or the other could be the donor excited state. Examination of the mutual orientations of Trp-14, Trp-25, and Trp-45 from the crystal structures of sTF (Muller et al., 1994; Muller et al., 1996; Harlos et al., 1994) indicates that either Trp-14 or Trp-25 could act as an energy transfer donor to Trp-45, accounting for the non-additivity of the 5F-Trp difference spectra.

To assess the possibility of RET from sites 14 and/or 25 to 45, we used the W45F fluorescence spectrum to represent the donor emission for the overlap integral calculation rather than the W14F or W25Y difference spectra for the following reasons. First, W14F and W25Y, which lack 5F-Trp at sites 14 and 25, respectively, have similar shapes and intensities. This suggested the *shape* of the emission spectrum of 5F-Trp would be approximately the same for either site 14 or 25. Second, if both sites are involved in RET, the relative fluorescence for site 45 will be greater in the wild type protein than in either W14F or W25Y because one (potential) donor is eliminated in each mutant. As a result, the difference spectra generated from the W14F and W25Y fluorescence will include a significant contribution from site 45, which would produce red-shifted difference spectra. In fact, the actual difference spectra are red-shifted significantly compared to the spectrum of W45F (see Figures 3-5 and 3-6). The calculated overlap integral for the W45F spectrum, corrected for the wavelength response of the fluorometer, with the single-Trp mutant difference absorption spectrum representing 5F-Trp at site 45 is $1.66 \times 10^{-16}$ $\mathrm{cm}^6\ \mathrm{mmol}^{-1}$ (Figure 3-8, Table 2). With this overlap integral, assuming random orientation of transition dipoles and a donor quantum yield of 0.1 (Schiller, 1972) in the absence of RET, RET can occur with 50% efficiency at distances of about 12 Å (Table 2), confirming the possibility for RET from either site 14 or 25 to site 45.

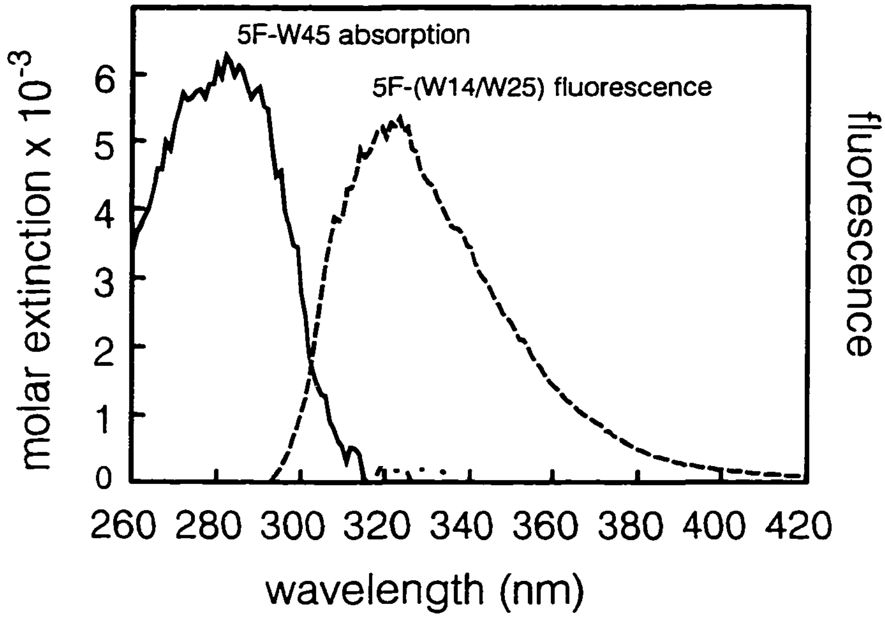


Figure 3-8: Overlap of the Trp 14 and Trp 25 fluorescence emission spectra with the Trp 45 absorbance spectrum.

### Utility of 5F-Trp as a Fluorescence Probe

The absorbance spectrum of 5F-Trp is red-shifted relative to the Trp absorbance spectrum, and the wavelength region from 300 to 310 nm provides a window in which 5F-Trp can be selectively excited in the presence of Trp. Additionally, based on our results for the spectroscopy of single-Trp replacement mutants, trends similar but not identical to Trp are observed for environment-dependent shifts of fluorescence and changes in quantum yield of 5F-Trp fluorescence. In particular, residues that are non-fluorescent with indole as the side chain can become significantly fluorescent when 5F-indole is the side chain. This needs to be taken into account in understanding the fluorescence of an analog containing protein. Also, whereas additivity is observed for the Trp difference fluorescence spectra of the single-Trp replacement mutants (Hasselbacher et al., 1995a), the 5F-Trp difference spectra do not sum to yield the wild type protein spectrum. However, inter-residue RET can rationalize the results for 5F-Trp fluorescence.

The spectroscopic features of 5F-Trp and the ease of its incorporation in recombinant proteins suggest its utility as a probe for studies akin to those that have been performed with other Trp analogs that display red-shifted spectra, specifically 5OH-Trp and 7A-Trp (Ross et al., 1997). Although, these other analogs have greater extinction in the red-shifted portions of their absorbance spectra than 5-Trp, the indole ring substitutions of these analogs either are bulkier (5OH-Trp) or alter hydrogen bonding (7A-Trp), and thus are potentially more perturbing to the local structure. In particular, we have demonstrated here with sTF that a protein that cannot tolerate 5OH-Trp and 7A-Trp can tolerate the less bulky 5F-Trp. Thus, we anticipate that 5F-Trp will be a satisfactory alternative to enhance the spectroscopic properties of other proteins that cannot tolerate 5OH-Trp or 7A-Trp.

## REFERENCES:

Barlati, S. and O. Ciferri. 1970. Incorporation of 5-Methyl- and 5-Hydroxy-Tryptophan into the Protein of *Bacillus subtilis*. *J. Bacteriol.* 101:166-172.

Bom, V.J. and R.M. Bertina. 1990. The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. *Biochem. J.* 265:327-336.

Bronskill, P.M. and J.T. Wong. 1988. Suppression of fluorescence of tryptophan residues in proteins by replacement with 4-fluorotryptophan. *Biochem. J.* 249:305-308.

Broze, G.J., Jr. and P.W. Majerus. 1980. Purification and properties of human coagulation factor VII. *J. Biol. Chem.* 255:1242-1247.

Callis, P.R. 1997. $^1L_a$ and $^1L_b$ Transitions of Tryptophan: Applications of Theory and Experimental Observations to Fluorescence of Proteins. In Fluorescence Spectroscopy. L. Brand and M.L. Johnson, editors. Academic Press, New York. 113-150.

Danielson, M.A. and J.J. Falke. 1996. Use of 19F NMR to Probe Protein Structure and Conformational Changes. In Annual Review of Biophysics and Biomolecular Structure.

R.M. Stroud, W.L. Hubbel, W.K. Olson, and M.P. Sheetz, editors. Annual Reviews, Inc. Palo Alto. 163-195.

Edgington, T.S., C.D. Dickinson, and W. Ruf. 1997. The Structural Basis of Function of the TF-VIIa Complex in the Cellular Initiation of Coagulation. *Thromb. Haemost.* 78:401-405.

Eisinger, J., B. Feuer, and A.A. Lamola. 1969. Intramolecular Singlet Excitation Transfer. Applications to Peptides. *Biochemistry* 8:3908-3915.

Eftink, M.R. 1991. Fluorescence Techniques for Studying Protein Structure. *Meth. Biochem. Anal.* 35:127-205.

Förster, T. 1948. Intermolecular Energy Transfer and Fluorescence. *Ann. Physik* 2:55-75.

Harlos, K., D.M. Martin, D.P. O'Brien, E.Y. Jones, D.I. Stuart, I. Polikarpov, A. Miller, E.G. Tuddenham, and C.W. Boys. 1994. Crystal structure of the extracellular region of human tissue factor [published erratum appears in Nature 1994 Oct 20;371(6499): 720]. *Nature* 370:662-666.

Hasselbacher, C.A., E. Rusinova, E. Waxman, R. Rusinova, R.A. Kohanski, W. Lam, A. Guha, J. Du, T.C. Lin, I. Polikarpov, and et al., 1995a. Environments of the four tryptophans in the extracellular domain of human tissue factor: comparison of results from absorption and fluorescence difference spectra of tryptophan replacement mutants with the crystal structure of the wild-type protein. *Biophys. J.* 69:20-29.

Hasselbacher, C.A., R. Rusinova, E. Rusinova, and J.B.A. Ross. 1995b. Spectral Enhancement of Recombinant Proteins with Tryptophan Analogs: The Soluble Domain of Human Tissue Factor. In Techniques in Protein Chemistry. J.W. Crabb, editor. Academic Press, New York. 349-356.

Hogue, C.W., I. Rasquinha, A.G. Szabo, and J.P. MacManus. 1992. A new intrinsic fluorescent probe for proteins. Biosynthetic incorporation of 5-hydroxytryptophan into oncomodulin. *FEBS Lett.* 310:269-272.

Kirchhofer, D. and Y. Nemerson. 1996. Initiation of blood coagulation: the tissue factor/factor VIIa complex. *Curr. Opin. Biotechnol.* 7:386-391.

Lark, K.G. 1969. Incorporation of 5-Methyltryptophan into the Protein of *Escherichia coli* I5 T-(555-7). *J. Bacteriol.* 97:980-982.

Laue, T.M., D.F. Senear, S. Eaton, and J.B. Ross. 1993. 5-hydroxytryptophan as a new intrinsic probe for investigating protein-DNA interactions by analytical ultracentrifugation. Study of the effect of DNA on self-assembly of the bacteriophage lambda cI repressor. *Biochemistry* 32:2469-2472.

Longworth, J.W. 1971. Luminescence of Polypeptides and Proteins. In Excited States of Proteins and Nucleic Acids. R.F. Steiner and I. Weinryb, editors. Plenum Press, New York. 319-484.

Miletich, J.P., G.J. Broze,Jr., and P.W. Majerus. 1981. Purification of human coagulation factors II, IX, and X using sulfated dextran beads. *Methods Enzymol.* 80:221-228.

Muller, Y.A., M.H. Ultsch, and A.M. de Vos. 1996. The crystal structure of the extracellular domain of human tissue factor refined to 1.7 A resolution. *J. Mol. Biol.* 256:144-159.

Muller, Y.A., M.H. Ultsch, R.F. Kelley, and A.M. de Vos. 1994. Structure of the extracellular domain of human tissue factor: location of the factor VIIa binding site. *Biochemistry* 33:10864-10870.

Nemerson, Y. and R. Gentry. 1986. An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage [published erratum appears in Biochemistry 1987 Feb 10;26(3):974]. *Biochemistry* 25:4020-4033.

Østerud, B. 1997. Tissue Factor: a complex biological role. *Thromb. Haemost.* 78:755-758.

Pardee, A.B., V.G. Shore, and L.S. Prestidge. 1956. Incorporation of Azatryptophan into Proteins of Bacteria and Bacteriphage. *Biochim. Biophys. Acta* 21:406-407.

Pratt, F.A. and C. Ho. 1975. Incorporation of Fluorotryptophan in Proteins of *Escherichia coli. Biochemistry* 14:3035-3040.

Ross, J.B.A., D.F. Senear, E. Waxman, B.B. Kombo, E. Rusinova, Y.T. Huang, W.R. Laws, and C.A. Hasselbacher. 1992. Spectral enhancement of proteins: biological incorporation and fluorescence characterization of 5-hydroxytryptophan in bacteriophage lambda cI repressor. *Proc. Natl. Acad. Sci. U. S. A.* 89:12023-12027.

Ross, J.B.A., A.G. Szabo, and C.W.V. Hogue. 1997. Enhancement of Protein Spectra with Tryptophan Analogs: Fluorescence Spectroscopy of Protein-Protein and Protein-Nucleic Acid Interactions. In Fluorescence Spectroscopy. L. Brand and M.L. Johnson, editors. Academic Press, New York. 151-190.

Ruf, W. and T.S. Edgington. 1994. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. *FASEB J.* 8:385-390.

Ruf, W., A. Rehemtulla, J.H. Morrissey, and T.S. Edgington. 1991. Phospholipid-independent and -dependent interactions required for tissue factor receptor and cofactor function [published erratum appears in J Biol Chem 1991 Aug 25;266(24):16256]. *J. Biol. Chem.* 266:2158-2166.

Schiller, P.W. 1972. Study of Adrenocorticotropic Hormone Conformation by Evaluation of Intramolecular Resonance Energy Transfer in $N^{\epsilon}$-Dansyllysine-ACTH-(1-24)-Tetrakosipeptide. *Proc. Nat. Acad. Sci.* 69:975-979.

Schlesinger, S. 1968. The Effect of Amino Acid Analogues on Alkaline Phosphatase Formation in *Escherichia coli* K-12. *J. Biol. Chem.* 243:3877-3883.

Shen, F., S.J. Triezenberg, P. Hensley, D. Porter, and J.R. Knutson. 1996. Transcriptional activation domain of the herpesvirus protein VP16 becomes conformationally constrained upon interaction with basal transcription factors. *J. Biol. Chem.* 271:4827-4837.

Soumillion, P., L. Jespers, J. Vervoort, and J. Fastrez. 1995. Biosynthetic incorporation of 7-azatryptophan into the phage lambda lysozyme: estimation of tryptophan accessibility, effect on enzymatic activity and protein stability. *Protein Eng.* 8:451-456.

Waxman, E., E. Rusinova, C.A. Hasselbacher, G.P. Schwartz, W.R. Laws, and J.B.A. Ross. 1993. Determination of the tryptophan:tyrosine ratio in proteins. *Anal. Biochem.* 210:425-428.

Wong, C.Y. and M.R. Eftink. 1997. Biosynthetic incorporation of tryptophan analogues into staphylococcal nuclease: effect of 5-hydroxytryptophan and 7-azatryptophan on structure and stability. *Protein Sci.* 6:689-697.

Zemsky, J., E. Rusinova, Y. Nemerson, L.A. Luck, and J.B.A. Ross. 1998. $^{19}$F NMR of 5-Fluorotryptophan in Soluble Human Tissue Factor: Comparison with Results from Tryptophan Fluorescence. *Biophys. J.* , submitted.

# CHAPTER 4

## Summary

The goals of this study were two fold. The first goal was to establish the utility of 5F-Trp as a probe of protein structure in fluorescence and $^{19}$F NMR studies. The second goal was to generate a spectrally enhanced version of sTF to allow further characterization of the sTF-VIIa complex. To accomplish these goals, 5F-Trp containing sTF protein and single Trp replacement mutant proteins of sTF were expressed in a Trp auxotroph of *E. coli.*

**Incorporation of 5F-Trp into sTF:**

In order to use 5F-Trp as a probe, it was first necessary to ascertain if 5F-Trp could be incorporated into protein at high levels of replacement. While 5F-Trp has been widely used as a $^{19}$F NMR probe in proteins, the levels of incorporation have varied widely (Danielson and Falke, 1996; Gerig, 1994). To be useful as a fluorescence probe, 5F-Trp must be incorporated to a high percent of the total Trp present.

The first part of chapter 2 examined the expression of 5F-Trp containing proteins in a Trp auxotroph of *E. coli*, the effect of 5F-Trp on cofactor activity and analyzed the level of 5F-Trp incorporation into protein. 5F-Trp was not found to alter the expression pattern of either the wild type or the single Trp replacement mutants relative to expression in the presence of Trp. Furthermore, 5F-Trp was found to have no effect on cofactor function by sTF or any of the mutants. Incorporation of 5F-Trp was analyzed using two approaches, LINCS analysis (Waxman et al., 1993) and mass spectrometry. Both methods indicated that 5F-Trp could be incorporated to high levels in sTF and the single Trp replacement mutants. These results contrast with previous sTF studies which found that Trp analogs (5OH-Trp and 7A-Trp) yielded insoluble, poorly incorporated proteins (Hasselbacher et al., 1995b).

The second question relating to the incorporation of 5F-Trp was the efficiency of incorporation. Two different approaches, LINCS analysis (Waxman et al., 1993) and mass spectrometry measured incorporation. Both methods yielded similar proportions of 5F-Trp in each protein. 5F-Trp was found to replace from 77 to nearly 100% of the total Trp present. Efficiency of replacement was greater for the wild type than for the mutants. The reason for this difference is unknown.

### Characterization of sTF by $^{19}$F NMR:

The $^{19}$F NMR spectrum of sTF displayed four peaks, two of which overlapped. Each peak was of equal intensity indicating that each 5F-Trp residue was represented and that 5F-Trp had incorporated equally into each site. Three of the single Trp replacement mutants were used to identify which peak correlated with each Trp residue. As with the wild type, 5F-Trp replacement of Trp was found to be equal among sites. In addition to the correlation of the peaks with the correct Trp residues, the mutant spectra revealed that substitutions in Domain I of sTF result in alterations of the local environment. However, these alterations do not appear to have a significant effect on protein function, as all the mutants attain the same $V_{max}$ as the wild type.

The wild type and mutant proteins were further characterized using gadolinium chloride, a paramagnetic line broadening agent. Gadolinium can only broaden the peaks of those residues which are solvent exposed. The data on solvent exposure was compared with that of the previous study, examining the difference fluorescence spectra of Trp sTF (Hasselbacher et al., 1995a) and were found not to entirely correlate with each other. The fluorescence data indicated that both Trp 45 and Trp 158 were solvent exposed. However, only the peak correlating with Trp 158 broadened significantly upon addition of gadolinium. The difference is a result of what part of the molecule is being monitored. Fluorescence emission spectra report on the environment of the indole ring as a whole, while $^{19}$F NMR reports only on the environment of the fluorine atom. Examined together, the results correlate well with the reported crystal structures which report that both residues are located at the surface of the molecule, but that position 5 of the indole ring of Trp 45 is buried while that of Trp 158 is solvent exposed (Harlos et al., 1994; Muller et al., 1994).

**Absorbance and Fluorescence Characteristics of 5F-Trp:**

In Chapter 3, the absorbance and fluorescence emission characteristics of free 5F-Trp and 5F-Trp in sTF were studied. While 5F-Trp has been used extensively in $^{19}$F NMR studies, very little was known about the absorbance and fluorescence characteristics of this probe. The absorption spectrum of 5F-Trp was found to have greater extinction than that of Trp over the entire range from 260 to 340 nm. In particular, 5F-Trp has significant extinction from 300 to 310 nm while Trp has essentially no extinction. This difference is the basis for using 5F-Trp to generate spectrally enhanced proteins. These results correlated well with previous observations of the 5F-Trp spectrum (Bronskill and Wong, 1988).

Fluorescence emission spectra of 5F-Trp and Trp in both pH 7.4 buffer and dioxane were measured, exciting the amino acids at 280 nm. The 5F-Trp emission spectra responded identically to the Trp emission spectra to the different environments. In both pH 7.4 buffer and dioxane, 5F-Trp is approximately 4 nm red-shifted relative to Trp. These results suggest that 5F-Trp has a similar sensitivity to environment in terms of its polarity as Trp and will, therefore, be useful as a probe of local environment.

The fluorescence emission spectra of 5F-Trp sTF and the single Trp replacement mutants of sTF were measured by exciting the proteins at 295 nm. These data were compared with the fluorescence emission spectra of Trp sTF and Trp single replacement mutants characterized in the previous study (Hasselbacher et al., 1995a). Previously it was observed that Trp 14 and Trp 45 accounted for the entire fluorescence spectrum of sTF. In contrast, when 5F-Trp is present, three Trp sites contribute to the fluorescence spectrum. The W45F mutant was noted to have an extremely blues shifted spectrum. Because three of the residues are fluorescent and the 5F-Trp absorbance and fluorescence emission spectra have greater overlap than those of Trp, resonance energy transfer may be occurring between the more buried residues and 5F-Trp 45. These residues would not be quenched in the absence of 5F-Trp 45, resulting in a very blue spectrum. However, the possibility of resonance energy transfer and the blue spectrum of W45F make generation of accurate difference spectra impossible. The relative contributions of Domain I residues, which are close enough to each other to participate in energy transfer, would be incorrect in all mutants except for W158F. It is, therefore, not possible to definitively state that the difference spectra are additive, although it is likely.

**Future Directions:**

Both the general spectral characteristics of 5F-Trp and the specific characteristics of 5F-Trp sTF were of interest in this study. 5F-Trp appears to have several characteristics that suggest that it would indeed be useful for generating spectrally enhanced proteins. The usefulness of 5F-Trp as a $^{19}$F NMR probe has been well documented. The red-shifted absorbance spectrum of 5F-Trp provides the means to selectively excite a 5F-Trp containing protein in the presence of a Trp containing protein, allowing characterization of one protein within a complex. These two methods used together would provide a highly sensitive tool with which to probe local environments, yielding information on both the environment of the indole ring and the fluorine atom. Furthermore, the increased overlap between the 5F-Trp absorbance and fluorescence spectra may make it useful for resonance energy transfer studies.

sTF has been studied extensively by multiple approaches. The crystal structure of the sTF-VIIa complex has been interpreted to suggest that sTF acts only as a scaffold for VIIa and is not involved in enhancement of VIIa activity (Banner et al., 1996). This conclusion is based upon the observation that the sTF backbone remains unchanged relative to the crystal structure of sTF alone. However, subtler alterations in the side chains of sTF residues may be occurring that enhance VIIa. Both fluorescence and $^{19}$F NMR spectroscopies are highly sensitive to changes in local environments in solution that may be missed in crystal structure analysis. The next step to probe for these types of alterations would be characterization of 5F-Trp sTF in complex with Trp VIIa.

## REFERENCES

Ashton, A.W., G. Kemball-Cook, D.J.D. Johnson, D.M.A. Martin, D.P. O'Brien, E.G. Tuddenham, and S.J. Perkins. 1995. Factor VIIa and the extracellular domains of human tissue factor form a compact complex: a study by X-ray and neutron solution scattering. *FEBS Lett.* 374:141-146.

Bach, R. 1988. Initiation of coagulation by tissue factor. *CRC Crit. Rev. Biochem.* 23:339-368.

Bach, R. and D.B. Rifkin. 1990. Expression of Tissue Factor Procoagulant Activity: Regulation by Cytosolic Calcium. *Proceedings of the National Academy of Science, USA* 87:6995-6999.

Bach, R., R. Gentry, and Y. Nemerson. 1986. Factor VII binding to tissue factor in reconstituted phospholipid vesicles: induction of cooperativity by phosphatidylserine. *Biochemistry* 25:4007-4020.

Banner, D.W. 1997. The Factor VIIa / Tissue Factor Complex. *Thromb. Haemost.* 78:512-515.

Banner, D.W., A. D'Arcy, C. Chene, F.K. Winkler, A. Guha, W.H. Konigsberg, Y. Nemerson, and D. Kirchhofer. 1996. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor [see comments]. *Nature* 380:41-46.

Barlati, S. and O. Ciferri. 1970. Incorporation of 5-Methyl- and 5-Hydroxy-Tryptophan into the Protein of *Bacillus subtilis*. *J. Bacteriol.* 101:166-172.

Bom, V.J. and R.M. Bertina. 1990. The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. *Biochem. J.* 265:327-336.

Bronskill, P.M. and J.T. Wong. 1988. Suppression of fluorescence of tryptophan residues in proteins by replacement with 4-fluorotryptophan. *Biochem. J.* 249:305-308.

Broze, G.J., Jr. and P.W. Majerus. 1980. Purification and properties of human coagulation factor VII. *J. Biol. Chem.* 255:1242-1247.

Callis, P.R. 1997. $^1L_a$ and $^1L_b$ Transitions of Tryptophan: Applications of Theory and Experimental Observations to Fluorescence of Proteins. In Fluorescence Spectroscopy. L. Brand and M.L. Johnson, editors. Academic Press, New York. 113-150.

Danielson, M.A. and J.J. Falke. 1996. Use of 19F NMR to Probe Protein Structure and Conformational Changes. In Annual Review of Biophysics and Biomolecular Structure. R.M. Stroud, W.L. Hubbel, W.K. Olson, and M.P. Sheetz, editors. Annual Reviews, Inc. Palo Alto. 163-195.

Davie, E.W. 1995. Biochemical and molecular aspects of the coagulation cascade. *Thromb. Haemost.* 74:1-6.

Drake, T.A., J. Cheng, A. Chang, and F.B. Taylor. 1993. Expression of Tissue Factor, Thrombomodulin, and E-Selectin in Baboons with Lethal *Escherichia coli* Sepsis. *American Journal of Pathology* 142:1457-1470.

Edgington, T.S., C.D. Dickinson, and W. Ruf. 1997. The Structural Basis of Function of the TF-VIIa Complex in the Cellular Initiation of Coagulation. *Thromb. Haemost.* 78:401-405.

Edgington, T.S., N. Mackman, K. Brand, and W. Ruf. 1991. The structural biology of expression and function of tissue factor. *Thromb. Haemost.* 66:67-79.

Eisinger, J., B. Feuer, and A.A. Lamola. 1969. Intramolecular Singlet Excitation Transfer. Applications to Peptides. *Biochemistry* 8:3908-3915.

Eftink, M.R. 1991. Fluorescence Techniques for Studying Protein Structure. *Meth. Biochem. Anal.* 35:127-205.

Fiore, M.M., P.F. Neuenschwander, and J.H. Morrissey. 1994. The biochemical basis for the apparent defect of soluble mutant tissue factor in enhancing the proteolytic activities of factor VIIa. *J. Biol. Chem.* 269:143-149.

Förster, T. 1948. Intermolecular Energy Transfer and Fluorescence. *Ann. Physik* 2:55-75.

Gerig, J.T. 1994. Fluorine NMR of Proteins. *Prog. NMR Spectrosc.* 26:293-370.

Harlos, K., D.M. Martin, D.P. O'Brien, E.Y. Jones, D.I. Stuart, I. Polikarpov, A. Miller, E.G. Tuddenham, and C.W. Boys. 1994. Crystal structure of the extracellular region of human tissue factor [published erratum appears in Nature 1994 Oct 20;371(6499): 720]. *Nature* 370:662-666.

Haskel, E.J., S.R. Torr, K.C. Day, M.O. Palmier, T.C. Wun, B.E. Sobel, and D.R. Abendschein. 1991. Prevention of arterial reocclusion after thrombolysis with recombinant lipoprotein-associated coagulation inhibitor [see comments]. *Circulation* 84:821-827.

Hasselbacher, C.A., E. Rusinova, E. Waxman, R. Rusinova, R.A. Kohanski, W. Lam, A. Guha, J. Du, T.C. Lin, I. Polikarpov, and Ross. J.B.A. 1995. Environments of the four tryptophans in the extracellular domain of human tissue factor: comparison of results from absorption and fluorescence difference spectra of tryptophan replacement mutants with the crystal structure of the wild-type protein. *Biophys. J.* 69:20-29.

Hasselbacher, C.A., R. Rusinova, E. Rusinova, and J.B.A. Ross. 1995. Spectral Enhancement of Recombinant Proteins with Tryptophan Analogs: The Soluble Domain of Human Tissue Factor. In Techniques in Protein Chemistry. J.W. Crabb, editor. Academic Press, New York. 349-356.

Hogue, C.W. and A.G. Szabo. 1993. Characterization of aminoacyl-adenylates in B. subtilis tryptophanyl-tRNA synthetase, by the fluorescence of tryptophan analogs 5-hydroxytryptophan and 7-azatryptophan. *Biophys. Chem.* 48:159-169.

Hogue, C.W., I. Rasquinha, A.G. Szabo, and J.P. MacManus. 1992. A new intrinsic fluorescent probe for proteins. Biosynthetic incorporation of 5-hydroxytryptophan into oncomodulin. *FEBS Lett.* 310:269-272.

Hogue, C.W.V. 1994. Tryptophanyl-tRNA Synthetase and Its Role in the Incorporation of New Intrinsic Fluorescent Probes into Proteins. Dissertation, University of Ottawa. Ottawa, Canada.

Hott, J.L. and R.F. Borkman. 1989. The non-fluorescence of 4-fluorotryptophan. *Biochem. J.* 264:297-299.

Kirchhofer, D. and Y. Nemerson. 1996. Initiation of blood coagulation: the tissue factor/factor VIIa complex. *Curr. Opin. Biotechnol.* 7:386-391.

Lark, K.G. 1969. Incorporation of 5-Methyltryptophan into the Protein of *Escherichia coli* I5 T-(555-7). *J. Bacteriol.* 97:980-982.

Lau, E.Y. and J.T. Gerig. 1997. Effects of Fluorine Substitution on the Structure and Dynamics of Complexes of Dihydrofolate Reductase (*Escherichia coli*). *Biophysical Journal* 73:1579-1592.

Laue, T.M., D.F. Senear, S. Eaton, and J.B. Ross. 1993. 5-hydroxytryptophan as a new intrinsic probe for investigating protein-DNA interactions by analytical ultracentrifugation. Study of the effect of DNA on self-assembly of the bacteriophage lambda cI repressor. *Biochemistry* 32:2469-2472.

Longworth, J.W. 1971. Luminescence of Polypeptides and Proteins. In Excited States of Proteins and Nucleic Acids. R.F. Steiner and I. Weinryb, editors. Plenum Press, New York. 319-484.

Luck, L. A. and J.J. Falke. 1991. Open Conformation of a Substrate-Binding Cleft: $^{19}$F NMR Studies of Cleft Angle in the D-Galactose Chemosensory Receptor. *Biochemistry* 30:6484-6490.

McCallum, C.D., R.C. Hapak, P.F. Neuenschwander, J.H. Morrissey, and A.E. Johnson. 1996. The location of the active site of blood coagulation factor VIIa above the membrane surface and its reorientation upon association with tissue factor. A fluorescence energy transfer study. *J. Biol. Chem.* 271:28168-28175.

Miletich, J.P., G.J. Broze,Jr., and P.W. Majerus. 1981. Purification of human coagulation factors II, IX, and X using sulfated dextran beads. *Methods Enzymol.* 80:221-228.

Mueller, B.M., R.A. Reisfeld, T.S. Edgington, and W. Ruf. 1992. Expression of tissue factor by melanoma cells promotes efficient hematogenous metastasis. *Proc. Natl. Acad. Sci. U. S. A.* 89:11832-11836.

Muller. Y.A., M.H. Ultsch, and A.M. de Vos. 1996. The crystal structure of the extracellular domain of human tissue factor refined to 1.7 A resolution. *J. Mol. Biol.* 256:144-159.

Muller, Y.A., M.H. Ultsch, R.F. Kelley, and A.M. de Vos. 1994. Structure of the extracellular domain of human tissue factor: location of the factor VIIa binding site. *Biochemistry* 33:10864-10870.

Nemerson, Y. 1988. Tissue factor and hemostasis [published erratum appears in Blood 1988 Apr;71(4):1178]. *Blood* 71:1-8.

Nemerson, Y. 1995. Tissue factor: then and now. *Thromb. Haemost.* 74:180-184.

Nemerson, Y. and R. Gentry. 1986. An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage [published erratum appears in Biochemistry 1987 Feb 10;26(3):974]. *Biochemistry* 25:4020-4033.

Neuenschwander, P.F. and J.H. Morrissey. 1992. Deletion of the membrane anchoring region of tissue factor abolishes autoactivation of factor VII but not cofactor function. *J. Biol. Chem.* 267:14477-14482.

Neuenschwander, P.F. and J.H. Morrissey. 1995. Alteration of the substrate and inhibitor specificities of blood coagulation factor VIIa: importance of amino acid residue K192. *Biochemistry* 34:8701-8707.

Østerud, B. 1997. Tissue Factor: a complex biological role. *Thromb. Haemost.* 78:755-758.

Paborsky, L.R., I.W. Caras, K.L. Fisher, and C.M. Gorman. 1991. Lipid association, but not the transmembrane domain, is required for tissue factor activity. Substitution of the transmembrane domain with a phosphatidylinositol anchor. *J. Biol. Chem.* 266:21911-21916.

Pardee, A.B., V.G. Shore, and L.S. Prestidge. 1956. Incorporation of Azatryptophan into Proteins of Bacteria and Bacteriphage. *Biochim. Biophys. Acta* 21:406-407.

Pratt, F.A. and C. Ho. 1975. Incorporation of Fluorotryptophan in Proteins of *Escherichia coli*. *Biochemistry* 14:3035-3040.

Robertson, D.E., P.A. Kroon, and C. Ho. 1977. Nuclear Magnetic Resonance and Fluorescence Studies of Substrate-induced Conformational Changes of Histidine-binding Protein J of *Salmonella typhimurium*. *Biochemistry* 16:1443-1451.

Ross, J.B.A., A.G. Szabo, and C.W.V. Hogue. 1997. Enhancement of Protein Spectra with Tryptophan Analogs: Fluorescence Spectroscopy of Protein-Protein and Protein-Nucleic Acid Interactions. In Fluorescence Spectroscopy. L. Brand and M.L. Johnson, editors. Academic Press, New York. 151-190.

Ross, J.B.A., D.F. Senear, E. Waxman, B.B. Kombo, E. Rusinova, Y.T. Huang, W.R. Laws, and C.A. Hasselbacher. 1992. Spectral enhancement of proteins: biological incorporation and fluorescence characterization of 5-hydroxytryptophan in bacteriophage lambda cI repressor. *Proc. Natl. Acad. Sci. U. S. A.* 89:12023-12027.

Röttingen, J.A., T. Enden, E. Camerer, J.G. Iversen, and H. Prydz. 1995. Binding of human factor VIIa to tissue factor induces cytosolic Ca2+ signals in J82 cells, transfected COS-1 cells, Madin-Darby canine kidney cells and in human endothelial cells induced to synthesize tissue factor. *J. Biol. Chem.* 270:4650-4660.

Ruf, W. and T.S. Edgington. 1994. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. *FASEB J.* 8:385-390.

Ruf, W., A. Rehemtulla, J.H. Morrissey, and T.S. Edgington. 1991. Phospholipid-independent and -dependent interactions required for tissue factor receptor and cofactor function [published erratum appears in J Biol Chem 1991 Aug 25;266(24):16256]. *J. Biol. Chem.* 266:2158-2166.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Press, New York.

Sayle, R.A., and E.J. Milner-White. 1995. RasMol: Biomolecular graphics for all. *Trends in Biochemical Sciences.* 20:374-376.

Schiller, P.W. 1972. Study of Adrenocorticotropic Hormone Conformation by Evaluation of Intramolecular Resonance Energy Transfer in $N^{\varepsilon}$-Dansyllysine-ACTH-(1-24)-Tetrakosipeptide. *Proc. Nat. Acad. Sci.* 69:975-979.

Schlesinger, S. 1968. The Effect of Amino Acid Analogues on Alkaline Phosphatase Formation in *Escherichia coli* K-12. *J. Biol. Chem.* 243:3877-3883.

Semeraro, N. and M. Colucci. 1997. Tissue Factor in Health and Disease. *Thromb. Haemost.* 78:759-764.

Shen, F., S.J. Triezenberg, P. Hensley, D. Porter, and J.R. Knutson. 1996. Transcriptional activation domain of the herpesvirus protein VP16 becomes conformationally constrained upon interaction with basal transcription factors. *J. Biol. Chem.* 271:4827-4837.

Snavely, M.D., J.B. Florer, C.G. Miller, and M.E. Maguire. 1989. Magnesium transport in *Salmonella typhimurium*: expression of cloned genes for three distinct $Mg^{2+}$ transport systems. *J. Bacteriol.* 171:4752-4760.

Sørensen, B.B., E. Persson, P.-O. Freskgard, M. Kjalke, M. Ezban, T. Williams, and L.V.M. Rao. 1997. Incorporation of an active site inhibitor in factor VIIa alters the affinity for Tissue Factor. *J. Biol. Chem.* 272:11863-11868.

Soumillion, P., L. Jespers, J. Vervoort, and J. Fastrez. 1995. Biosynthetic incorporation of 7-azatryptophan into the phage lambda lysozyme: estimation of tryptophan accessibility, effect on enzymatic activity and protein stability. *Protein Eng.* 8:451-456.

Taubman, M.B., J.T. Fallon, A.D. Schecter, P. Giesen, M. Mendlowitz, B.S. Fyfe, J.D. Marmur, and Y. Nemerson. 1997. Tissue Factor in the Pathogenesis of Atherosclerosis. *Thromb. Haemost.* 78:200-204.

Waxman, E. 1993. Thermodynamic and hydrodynamic behavior of the Tissue Factor:Factor VII(a) complex. Dissertation, Mount Sinai School of Medicine of the City University of New York.

Waxman, E., E. Rusinova, C.A. Hasselbacher, G.P. Schwartz, W.R. Laws, and J.B.A. Ross. 1993. Determination of the tryptophan:tyrosine ratio in proteins. *Anal. Biochem.* 210:425-428.

Waxman, E., J.B.A. Ross, T.M. Laue, A. Guha, S.V. Thiruvikraman, T.C. Lin, W.H. Konigsberg, and Y. Nemerson. 1992. Tissue factor and its extracellular soluble domain: the relationship between intermolecular association with factor VIIa and enzymatic activity of the complex. *Biochemistry* 31:3998-4003.

Waxman, E., W.R. Laws, T.M. Laue, Y. Nemerson, and J.B.A. Ross. 1993. Human factor VIIa and its complex with soluble tissue factor: evaluation of asymmetry and conformational dynamics by ultracentrifugation and fluorescence anisotropy decay methods. *Biochemistry* 32:3005-3012.

Wilcox, J.N., K.M. Smith, S.M. Schwartz, and D. Gordon. 1989. Localization of Tissue Factor in the Normal Vessel Wall and in the Athersclerotic Plaque. *Proceedings of the National Academy of Science, USA* 86:2839-2843.

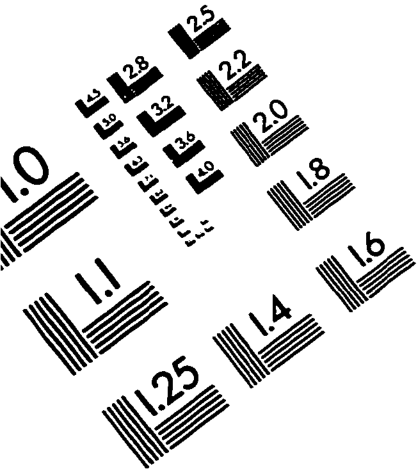
Wong, C.Y. and M.R. Eftink. 1997. Biosynthetic incorporation of tryptophan analogues into staphylococcal nuclease: effect of 5-hydroxytryptophan and 7-azatryptophan on structure and stability. *Protein Sci.* 6:689-697.

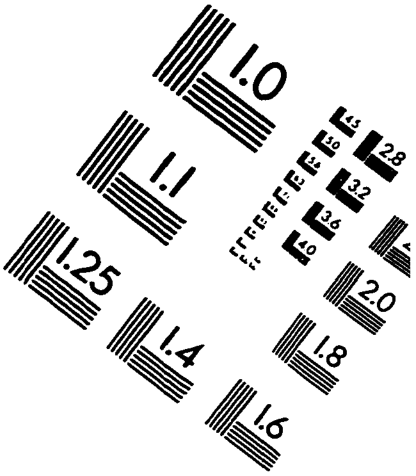
Xu, Z.J., M.L. Love, L.Y. Ma, M. Blum, P.M. Bronskill, J. Bernstein, A.A. Grey, T. Hofmann, N. Camerman, and J.T. Wong. 1989. Tryptophanyl-tRNA synthetase from Bacillus subtilis. Characterization and role of hydrophobicity in substrate recognition. *J. Biol. Chem.* 264:4304-4311.

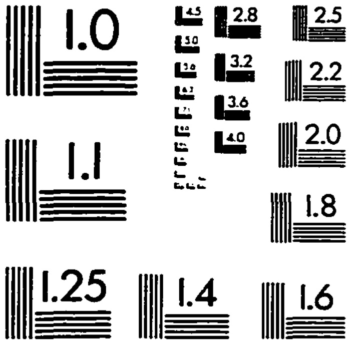
Zemsky, J., E. Rusinova, Y. Nemerson, L.A. Luck, and J.B.A. Ross. 1998. $^{19}$F NMR of 5-Fluorotryptophan in Soluble Human Tissue Factor: Comparison with Results from Tryptophan Fluorescence. *Biophys. J.* , submitted.

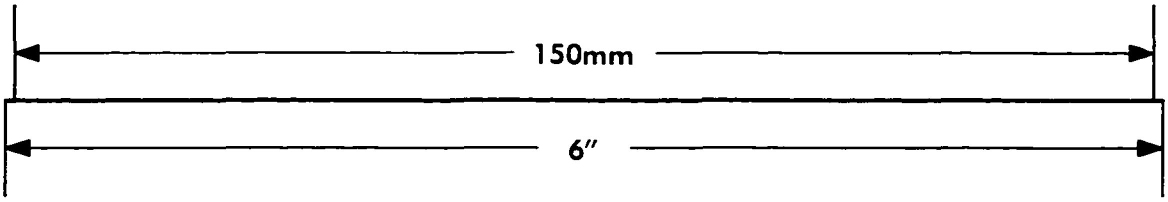
Zioncheck, T.F., S. Roy, and G.A. Vehar. 1992. The cytoplasmic domain of tissue factor is phosphorylated by a protein kinase C-dependent mechanism. *J. Biol. Chem.* 267:3561-3564.

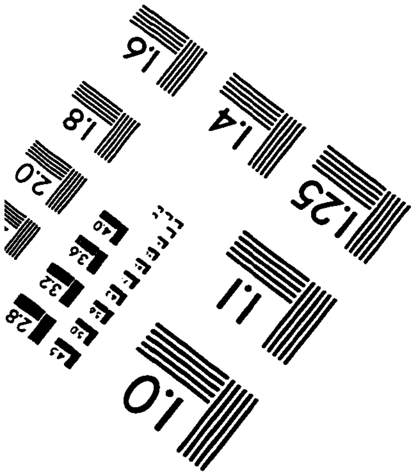
# IMAGE EVALUATION TEST TARGET (QA-3)

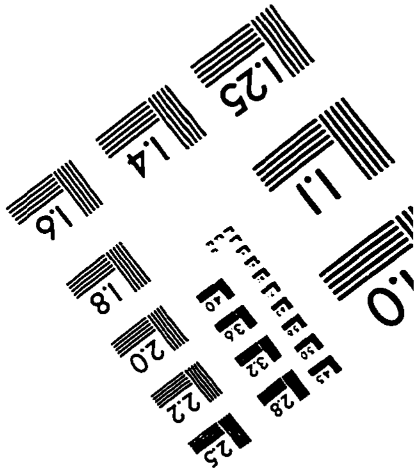












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