FLUORESCENCE AND X-RAY CRYSTALLOGRAPHIC STUDIES OF THE BINDING OF THE FLUORESCENT PROBE MOLECULE, ANS, TO CHYMOTRYPSIN

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This is to certify that the

thesis entitled

FLUORESCENCE AND X-RAY CRYSTALLOGRAPHIC STUDIES OF THE BINDING OF THE FLUORESCENT PROBE MOLECULE, ANS, TO CHYMOTRYPSIN

presented by

JOHN DAVID JOHNSON

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biophysics

M. A. El-Bayoumi

Major professor
M.A. El-Bayoumi

Date 1977

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ABSTRACT

FLUORESCENCE AND X-RAY CRYSTALLOGRAPHIC STUDIES OF THE BINDING OF THE FLUORESCENT PROBE MOLECULE, ANS, TO CHYMOTRYPSIN

By

John David Johnson

The fluorescent probe molecule, ANS (1-anilino-8-naphthalene sulfonate), binds to a distinct site, other than the active site on chymotrypsin in solution. Conformational changes occur about this site with the tryptic conversion of chymotrypsinogen to chymotrypsin and with changes in pH, which alter the fluorescence properties of bound ANS. Such changes in fluorescence allow us to monitor these protein conformational changes. In an effort to further characterize the ANS binding site on chymotrypsin and the fluorescence changes which occur with ANS binding to chymotrypsin and with changes in chymotrypsin conformation, we have conducted high resolution X-ray crystallographic studies of chymotrypsin-ANS crystals.

Correlation of our crystallographic and fluorescence studies shows that ANS binds to the same distinct site and enjoys the same environment in chymotrypsin crystals as in chymotrypsin solutions. Such fluorescence correlation crystallography has provided us with the first detailed "picture" of the binding of a fluorescence probe molecule to a protein.

ANS is bound to chymotrypsin near the cysteine 1-122 disulfide bridge. The conformation of bound ANS and its relation

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to the amino acid residues in its binding site are discussed in detail. The ANS binding site on chymotrypsin is not the typical hydrophobic binding site that such probes are traditionally thought to require for intense fluorescence. Mechanisms for ANS binding and for its intense fluorescence are considered.

The conformational changes that occur in chymotrypsin with pH changes have been detailed through the X-ray crystallographic analysis of Tulinsky and his co-workers. Examination of these studies revealed that conformational changes occur about the ANS binding site in chymotrypsin with pH. These changes are described and possible ways that these changes might alter the fluorescence of bound ANS are considered.

John David Johnson

FLUORESCENCE AND X-RAY CRYSTALLOGRAPHIC STUDIES OF THE BINDING OF THE FLUORESCENT PROBE MOLECULE, ANS, TO CHYMOTRYPSIN

By

John David Johnson

A DISSERTATION

submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biophysics

DEDICATION

To my family and my friends
who have given me more pride and happiness
than most people ever know

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ACKNOWLEDGEMENTS

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I greatly appreciate Larry Weber for his personal friendship, many valuable discussions and for his expertise and great expense of both time and energy in the crystallographic aspects of this work. The friendships, assistance and discussions of Joseph Kordas, David Carr, Sharmila Gupte and C. P. Chang are also greatly appreciated. The author is especially grateful to Elaine Johnston for her encouragement, friendship and understanding during the entire course of this work.

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INTRODUCTION

Because of the extreme sensitivity of their fluorescence to the polarity of their environment, fluorescence polarity probes such as 1-anilino-8-naphthalene sulfonate (ANS) and 2-p-toluidinyl-6-naphthalene sulfonate (TNS), have been used extensively to monitor biological phenomena at the molecular level. Typically, they are bound to a more or less specific site on a macromolecule or a macromolecular structure and exhibit a particular fluorescence, characteristic of their environment at that site. Structural changes in the macromolecule to which they are bound may often alter the probe's environment. This often results in rather dramatic changes in their fluorescence. Such fluorescence changes provide us with a convenient "handle" on many structural changes in biological systems.

To understand, at a molecular level, the structural change we are monitoring, we must know what factors are involved in determining the fluorescence properties of the probe. Ideally we would seek a probe molecule whose fluorescence is sensitive to only one parameter of its environment. Fluorescence changes in such a probe, when bound to a macromolecule, would then report to us, unambigously, the molecular change that occurs in this parameter in the vicinity of the probe.

In reality such ideal probes are seldom found. The best we may generally hope for is a probe whose fluorescence is

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sensitive to only a few environmental factors. The more completely we understand the factors which influence a probe's fluorescence, the more we know about the actual molecular changes that the probe is reporting.

Polarity probes such as ANS and TNS have fluorescence characteristics which appear to be particularly sensitive to one parameter: the ability of polar molecules to interact with them during the lifetime of their excited state. The greater the interaction of polar molecules with the probe during its excited state, the lower its fluorescence yield and its energy of emission.

Researchers initially saw this phenomena as a polarity effect. When ANS is in a nonpolar environment, there are less polar molecules to interact with its excited state and it fluoresces intensely. This lead to the belief that ANS and TNS fluoresce intensely, when bound to macromolecules, only when they are bound in a hydrophobic or nonpolar pocket or crevice in the macromolecular structure.

However, it is the ability of polar molecules to interact with the excited state and not merely the presence or absence of polar molecules in the environment of the probe that determines its fluorescence properties. While virtually non-fluorescent in liquid water, these probes fluoresce intensely in ice. Polar molecules are present in each case but only in liquid water do they have the ability to interact with the fluorophore during the lifetime of its excited state. This suggest that these probe molecules may fluoresce intensely even when bound to proteins at a polar site, if the polar molecules surrounding

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the bound probe are relatively immobile.

The use of fluorescence polarity probes, particularly ANS, has increased so dramatically since the pioneering studies of Weber,and Laurence and Stryer, that reviewers no longer even attempt to be comprehensive of their use. Because of the tremendous use of polarity probes in recent years and the wide number of biological phenomena they have probed on a molecular level, it is of vital importance to understand the mechanism of their fluorescence changes when bound to macromolecules.

We have correlated fluorescence studies in solutions of Chymotrypsin (CHT) and ANS with fluorescence and X-ray crystallographic studies of CHT-ANS crystals, under conditions where the complex is highly fluorescent. We had hoped that such studies would broaden our understanding of the molecular basis of ANS binding to proteins, the fluorescence enhancement when ANS is bound to proteins, and the fluorescence changes which occur with conformational changes in proteins.

X-ray crystallography is the only technique that allows the determination and examination of the complete three dimensional structure of proteins on a molecular level. It is also the only means of examinining the binding of a ligand, such as a fluorescent probe, at high resolution. We chose to study CHT because it is a readily available protein which has been extensively studied and especially because CHT crystals are easily grown and the crystal structure has been determined at high resolution by A. Tulinsky and his co-workers here at Michigan State University. We chose to study ANS because it is by far the most extensively used fluorescent probe molecule.

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I. INTRODUCTION TO EXCITED STATE PHENOMENA

A. Formation and Deactivation of the Excited State

Every chromophore has a series of closely spaced energy levels and may be raised from a lower to a higher energy state by the absorption of a discrete quantum of light of energy equal to the difference between these two energy levels. Molecules raised to these higher energy states are often capable of luminescence if their excited states are not depopulated by radiationless processes. Figure 1 represents the absorption process from various vibronic levels of the ground state, G, to various vibronic levels of the singlet state, S. Fluorescence occurs from the lowest vibrational level of the first excited singlet state, S_1, and phosphorescence from the triplet state, T_1.

Deactivation of the excited state of molecules may occur by several pathways. This is illustrated in Figure 2, taken from reference (1). Once excitation to a high singlet state such as S_3 occurs, a very rapid vibrational cascading process follows through the various vibrational levels of the excited state. In this radiationless process, known as internal conversion, the potential energy surface of S_3 is presumably cut or crossed by that of S_2. Thus some vibrational level of S_2 may be nearly isoenergetic with that of S_3. During the succeding vibrations it is highly likely that the molecule will vibrate in a manner corresponding to an energy state of S_2. As this cascading continues, the potential energy surface of S_1 may cut S_2. This process is repeated until internal conversion has taken the molecule to the lowest vibrational level of the lowest singlet excited state, S_1.

Figure 1

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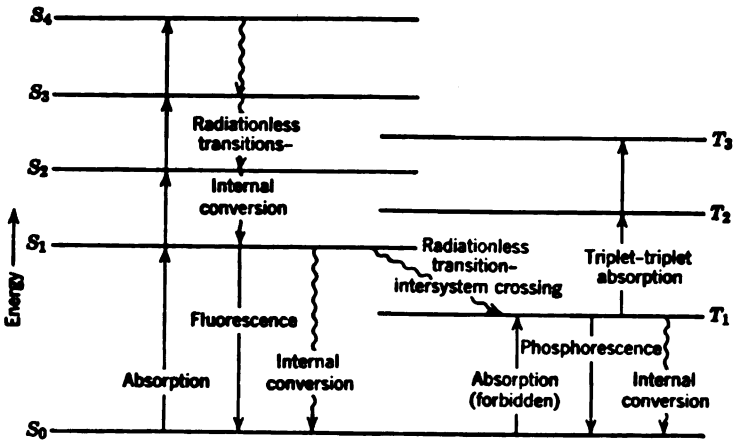

Figure 1 . Schematic state energy level diagram.

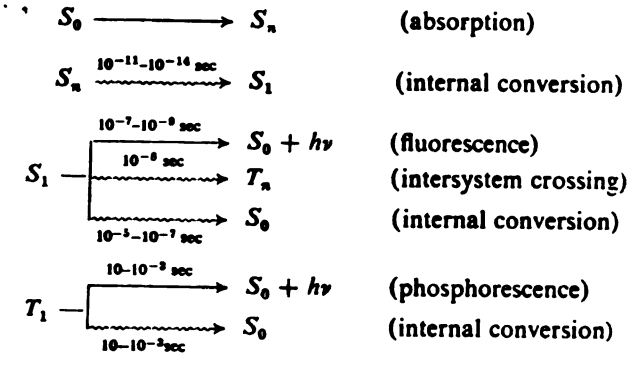

Figure 2 . Processes involved in the activation and deactivation of the excited state.

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Potential energy curves need not cross but only have to be sufficiently close to strongly interact or "mix". This provides the pathway for internal conversion. The collision of excited state molecules with solvent or other molecules may be responsible for part of the energy loss associated with internal conversion. Regardless of the mechanism involved, electronic energy is transformed and lost as vibrational energy through internal conversion until the lowest vibrational level of the lowest singlet state is obtained.

Since fluorescence nearly always occurs from this lowest vibrational level of the first singlet state, the probability of deactivation of higher singlet states by internal conversion is extremely high. Internal conversion may also occur from the lowest vibrational level of the lowest singlet state to the ground state, S_o . In this case it competes with other pathways of deactivation of the excited state and luminescence is reduced accordingly. However, complete quenching of fluorescence and phosphorescence by such internal conversion is rare.

Another radiationless transition may occur between the first excited singlet state and the lowest excited triplet state. This is known as intersystem crossing. The subsequent emission from the triplet state, known as phosphorescence, is characterized by its long lifetime and lower energy of emission. Radiationless deactivation of the triplet state by intersystem crossing to the ground state (S_o) is also possible and may compete with phosphorescence.

Certain of these processes (as diagramed in Figure 1) are of course , favored over others. Which process dominates depends

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on the fluorophor involved and its environment. Figure 2 represents a sequential outline of the processes concerned in the excitation and deactivation of a molecule. The approximate lifetimes of each state are given where relevant.

B. Solvent Effects on Absorption and Emission

Both the absorption and emission characteristics of fluorophores in solution are influenced by their environment. Such solvent effects are mediated by solvent interactions with the ground and/or excited state of the fluorescent solute.

The absorption of light is an electronic process which occurs on the order of 10^{-15}seconds. The electronic state immediately following the absorption process is the Franck-Condon state. In the Franck-Condon state the configuration of the solvent molecules about the solute is unchanged from their geometry in the ground state. Electronic rearrangements which occur instantaneously as a result of absorption may cause the dipole moment of the excited state molecule to change both in magnitude and direction, relative to the ground state dipole moment. Because of such changes, the configuration of solvent molecules around the ground state solute may not be the most stable configuration in the excited state. Relaxation of the solvent molecules may take place in times on the order of 10^{-11}seconds at room temperature. This may allow the reorientation of the solvent molecules to reach an equilibrium configuration which is compatable with the excited state dipole moment of the solute.

If instantaneous solute-solvent interactions stabilize the Franck-Condon state more than the ground state, then the absorption band corresponding to the transition between these two

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states will be red shifted to lower energies, relative to absorption in the vapor. If, however, solute-solvent interactions stabilize the ground state more than the Franck-Condon state during absorption, the absorption will be blue shifted to higher energies.

In the absorption process, the extremely short lifetimes of the Franck-Condon excited state limits the types of interactions which may occur during absorption and lower the energy of the primary absorption act. Relaxation to the equilibrium excited state configuration occurs rapidly before fluorescence. The lifetime of the fluorescent state is generally 10^{-8} to 10^{-9} seconds, which allows for the reorientation of solvent molecules about the excited state solute molecule.

When we consider solvent effects on fluorescence we are concerned with the electronic transition that occurs from the excited equilibrium state(where solvent-solute reorientation about the Franck-Condon excited state solute has occured) to the Franck-Condon ground state. Solvent effects on emission exert their influence through changes in the energy of the equilibrium excited state relative to the ground state. If such solvent-solute interactions can lower the energy of the equilibrium excited state more than the energy of the ground state, then a red shifted lower energy emission would be expected. If solvent-solute interactions lower the energy of the equilibrium excited state less than they lower the energy of the ground state, then a blue shifter higher energy emission would be expected. The interaction of the first excited singlet state with solvent molecules usually increases the probability

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of non-radiative processes. This may serve to decrease the fluorescence quantum yield.

These and other solvent effects are seen to drastically influence the fluorescence properties of many fluorophores, including two commonly used fluorescent probe molecules ANS and TNS. The utility of such fluorescent probe molecules lies in their ability to bind with high affinity to many proteins and macromolecules and the strong dependence of their fluorescence properties on their environment.

C. Characterization of the Excited State

Five physical parameters are generally used to characterize the excited state fluorescence of any molecule. These are the quantum yield, lifetime, energy, bandwidth and polarization of fluorescence.

Quantum yield: The quantum yield of fluorescence, Q_f, is a measure of the efficiency of the radiative process. It is

$$Q_f = \frac{\text{Number of quanta fluoresced}}{\text{Number of quanta absorbed}}$$

Since non-radiative processes such as intersystem crossing (ISC) and internal conversion (IC) may serve to depopulate the excited state and reduce the radiative rate constant k_r, the quantum yield is often expressed in terms of rate constants:

$$Q_f = \frac{k_r}{k_r + k_{IC} + k_{ISC}}$$

where k_{IC} and k_{ISC} are the rate constants for internal conversion and intersystem crossing from the first excited singlet state. Quantum yields range from zero, for non-fluorescent molecules,

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Lifetime: When a molecule is raised to an excited state by the absorption of a photon it will remain in that state until the excess energy is dissipated by fluorescence or other means of deactivation. The duration of a fluorophor in the fluorescent excited state is expressed in terms of its fluorescence lifetime. Lifetimes of organic molecules in solution are generally on the order of a few nanoseconds. While this is a short time to measure it is relatively long compared to the time required for many molecular processes.

Consider a sample being excited by continuous radiation. If there are N excited molecules providing a fluorescence intensity I, then when the excitation is removed, N and I decay exponentially : $I = I_o e^{-kt}$. Here, I_o is the fluorescence intensity at time t, and k is the rate constant for the decay. Thus the fluorescence lifetime is simply $\tau_f = 1/k$. In the absence of other decay processes, both external and internal, the radiative decay constant k is k_f and $\tau_o = 1/k_f$ is defined as the natural lifetime. The actual observed lifetime (τ_f) is determined by all deactivational processes and is expressed as $\tau_f = 1/k_{NR}$, where k_{NR} is the sum of all non-radiative processes. The relationship between the observed and natural lifetimes and quantum yield of fluorescence is $\tau_f = \tau_o Q_f = \tau_o(k_f/(k_f+k_{NR}))$. Experimentally τ_f and Q_f are measured and τ_f increases proportionally with Q_f. Thus the lifetime of the fluorescent state is directly related to the quantum yield of fluorescence and it also reflects changes in the radiative and non-radiative processes of an excited fluorophor.

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Energy: The energy of emission is the simplest of all of these parameters to determine. In any fluorescence spectrum the intensity of fluorescence is plotted as a function of wavelength (λ). The energy E associated with any wavelength of this spectrum can be determined by the expression $E = h\nu$, where ν is the frequency of the radiation and equals c/λ. Since fluorescence is emission from the lowest vibronic level of the first excited singlet state to the different energy levels of the ground state,such a spectrum may be rather broad. The parameter that is usually measured is the emission maxima (λ max), the wavelength at which the fluorescence intensity is a maximum.

Bandwidths: Bandwidths may also be used to further characterize an emission spectra. Generally the area under the emission spectrum is divided by the peak height or the widith of the spectrum at half peak height is used to determine the bandwidth.

Fluorescence polarization: Assume a molecule is excited by plane polarized light, propogated along the y-axis of a Cartesian coordinate system, with the electric vector vibrating in a specific direction, say parallel to the z-axis, as in Figure 3. If the fluorescence emission is observed through a polarizer along the x-axis, two values of emission intensity may be recorded: $I_{//}$ = the observed intensity when the polarizer is parallel to the plane of the electric vector of the exciting light and $I_{\perp}$ = the observed intensity when the polarizer is perpendicular to the plane of the electric vector of the exciting light. The polarization of fluorescence is then defined:

$$P = (I_{//} - I_{\perp})/(I_{//} + I_{\perp}).$$

If we excite a sample of fixed but randomly oriented

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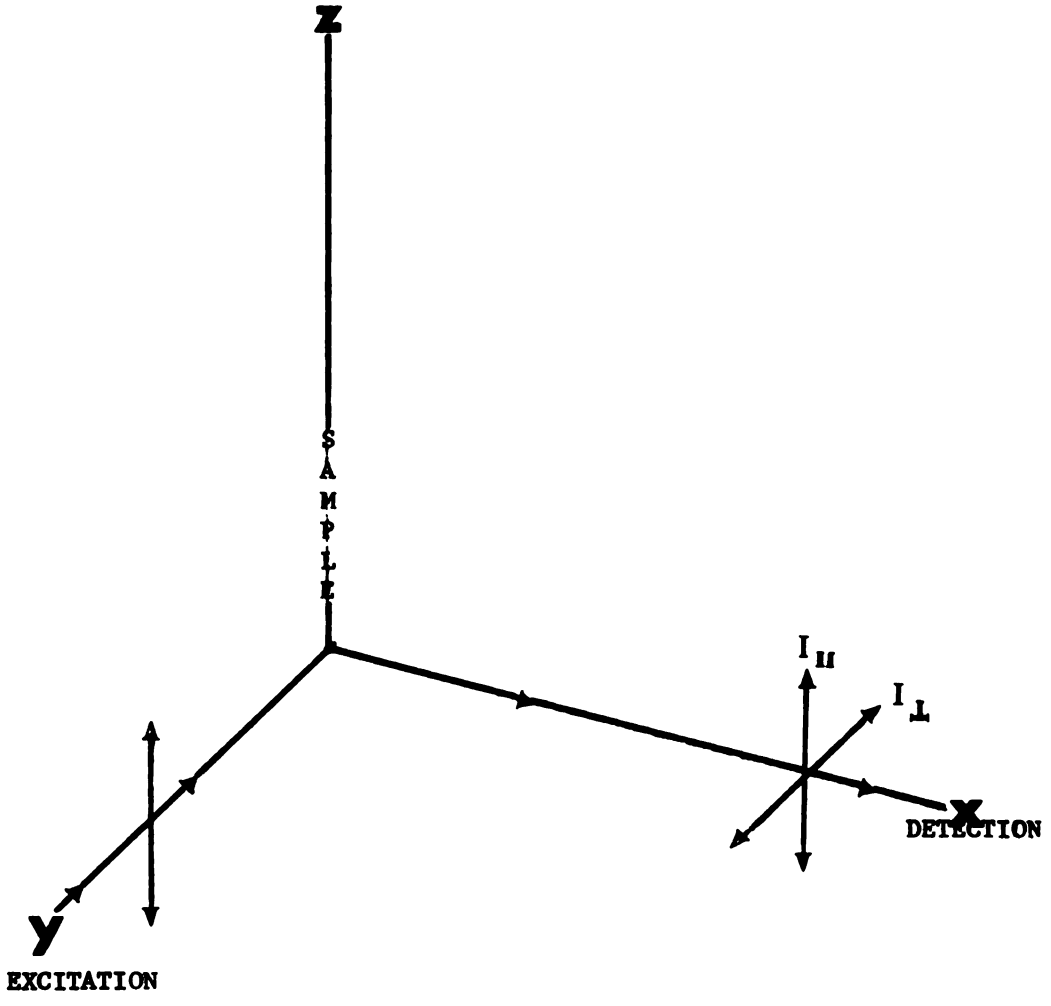

Figure 3 . Sample orientation with respect to emission and excitation polarizers in the determination of fluorescence polarization.

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fluorophores, those with their absorption dipoles parallel to the plane of polarized exciting light will be preferentially excited. This process is known as photoselection. The absorption and emission transition mements of a fluorophor have a definite spacial relationship to one another and to the axis of the molecule. While those molecules with their absorption transition moments parallel to the z-axis may be selectively excited emission will occur at some angle to the z-axis, depending on the relative oreintation of these absorption and emission dipole moments. The difference between the orientation of these transition moments gives rise to the $I_{\perp}$ component of the polarization.

Determination of the polarization of fluorescence under appropriate experimental conditions can yield information concerning the relative orientation of the absorption and emission dipole moments. The relative orientations of these dipole moments may be affected by changes in the environment of the fluorophor. Thus changes in the binding of a dye molecule to a protein may often be determined by changes in fluorescence polarization.

A sample in which the fluorophores are not fixed but can undergo rotation and Brownian motion may exhibit considerable depolarization of the exciting polarized light due to the rotation of the photoselected molecules between the time of absorption and emission. When a solution of fluorescent molecules is excited with polarized light the emission will be polarized to some extent. Brownian rotation of these molecules can sometime occur during the lifetime of the excited state and will serve

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to randomize the orientation of the excited molecules. This will decrease the degree of polarization. The degree of Brownian rotation depends on the size and shape of the molecule and the viscosity of the medium it is in. It may be determined by measuring the degree of depolarization as a function of time.

In practice, the fluorescence decay curves of the parallel and perpendicular components are measured in the nanosecond time range and the anisotropy is determined as a function of time: $A(t) = \left[I(t)_{//} - I(t)_{\perp}\right] / \left[I(t)_{//} + 2I(t)_{\perp}\right]$. A(t) is a measure of the change in the orientation of the emission transition moment between the time of absorption and the time of emission.

For a rigid sphere rotation, the anisotropy is $A(t) = A_o e^{-3t/\rho}$, where A_o is the initial anisotropy and depends on the relative orientations of the absorption and emission dipole moments, ρ is the rotational relaxation time and t is time. For a rigid sphere revolving, a plot of log A(t) versus time should yield a straight line with a slope equal to $-3/\rho$. Large molecules or molecules in a more viscous medium rotate more slowly. These factors are related to the rotational relaxation time ρ: $\rho = 3NV/kT$, where V is the volume of the hydrated sphere, N is the viscosity of the medium, k is Boltzman's constant and T is the absolute temperature. Changes in ρ can give us valuable information concerning the size, shape and flexibility of macromolecules which contain or bind fluorophores.

II. FLUORESCENCE AND SOLVENT POLARITY

A. Solvent Polarity

Before considering the effects of solvent polarity on

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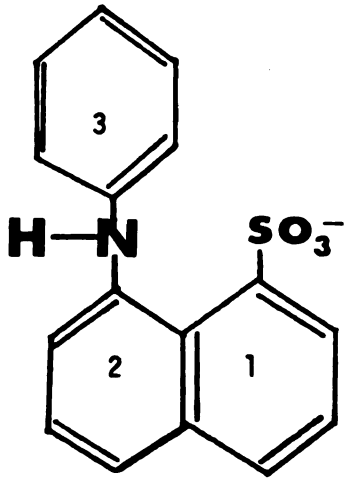
fluorophors, it is necessary to understand or at least to define what is meant by polarity. For our definition of polarity we accept the formulation of Reichardt (2). He considers polarity to include all the molecular interactions which a solute and solvent may undergo such as dispersion forces, dipole-dipole interactions and dipole-induced dipole interactions. Ionization and complex formation, which change the nature of the solute are excluded.

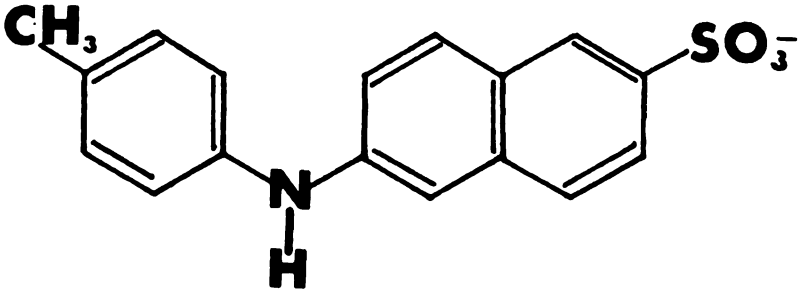
While the dielectric constant of the medium gives a good indication of polarity, empirical polarity scales (such as Kosower's) give a more reasonable approximation of polarity than macroscopic parameters such as static dielectric constants. The empirical (Z) solvent polarity scale of Kosower is based on the transition energy for the charge-transfer absorption for 1-ethyl-4-carboxymethoxypyridinium iodide in a particular solvent. This transition is highly sensitive to polarity. Such a polarity scale, of course, encompasses the dielectric constant. This polarity scale is often used as a measure of polarity. Its origin and application to other empirical solvent polarity scales is discussed in detail by Kosower (3).

B. Fluorescent Probes of Polarity

ANS and TNS are two commonly used probes of polarity. Their structures are shown in Figure 4. These structurally similar molecules are commonly refered to as "polarity probes" because their emission properties are extremely sensitive to the polarity of their environment (4,5,6,7). This sensitivity is demonstrated in Table 1, which shows the fluorescence quantum yields and emission wavelength maxima for ANS and TNS in alcohols



ANS



TNS

Figure 4 . The molecular structures of ANS and TNS .

	water	ethylene glycol	methanol	ethanol	n-propanol	n-butanol
ANS						
Q_F	.004	.15	.22	.37	.46	.56
λ max.	515	484	476	468	466	464
TNS						
Q_F	.0008	.14	.34	.52	.57	.57
λ max.	500	466	443	429	424	423

Table 1. The fluorescence yields and emission maxima of ANS and TNS in various alcohols. Taken from references (4,6).

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of various polarities and in aqueous solution. As the polarity of the alcohol decreases, the quantum yield of both ANS and TNS fluorescence increases dramatically as their fluorescence emission maxima blue shift to higher energies. Similar fluorescence changes occur as the percentage of ethanol or dioxane is increased in water mixtures of ANS or TNS. Thus in a polar medium such as water these fluorophors are virtually non-fluorescent yet in a non-polar solvent they are highly fluorescent at high energies.

Only minor changes in the absorption spectra of these fluorophores are observed in solutions of water and ethyl alchol, while these solvents elicit very different quantum yields. This suggest that ground state interactions play only a minor role in determining the fluorescence characteristics of these molecules.

C. Towards a Molecular Mechanism of Solvent Effects on ANS and TNS Emission

Detailed studies of the effects of various solvents, temperature, and chemical groups on the fluorescence parameters of these dyes and their derivatives has lead to a more complete understanding of solvent effects on their emissions. Through such studies the factors which effect their fluorescence yields, emission maxima and spectral bandwidths and the probable mechanism of such solvent induced fluorescence changes have become more throurghly understood.

Solvent Dependence of the Emission Maxima or Energy of the Fluorescence State. The molecular mechanism of solvent effects on the emission maxima or energy of the fluorescence of ANS and TNS is thought to be fairly well understood. The dipole orientation mechanism of Lippert (8) provides an explanation for the decrease

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in the emission maxima with decreasing solvent polarity. ANS and TNS have much higher dipole moments in their excited states than in their ground states. For example, the dipole moment in the first excited singlet state has been calculated to be 10 Debye greater than in the ground state for TNS (6). Excited state molecules would be expected to interact more strongly with polar or polarizable solvents because they are more polar than ground state molecules. This interaction would effectively lower the energy of the excited state molecules more than the energy of ground state molecules. An increase in the emission wavelength maxima would result from this lower energy emission.

Using nanosecond time resolved emission spectroscopy of ANS in various solvents, Chakrabarti and Ware (9) found support for the dipole orientation mechanism for the solvent dependence of the ANS emission maxima. They have observed spectral shifts to the red (lower energy) of ANS fluorescence in the nanosecond time range (2-68 nanoseconds) after excitation. These time dependent spectral shifts are due to the reorientation of solvent molecules (in the nanosecond time range) about the excited state ANS molecule. Such solvent reorientation is required to accomodate the change in the direction and magnitude of the dipole moment that occurs upon excitation. The degree to which such time dependent spectral shifts occur is a measure of the degree of relaxation of the excited state molecule to its equilibrium configuration. Similar studies of these spectral shifts in the emission maxima of TNS in the nanosecond time range in various solvents also lend support to the dipole orientation mechanism(10).

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Solvent Dependence of the Bandwidth. The increase in bandwidth of the emission spectra in more polar solvents may also be explained by the greater interaction of polar solvents with the excited state molecule. The larger number of interactions of polar molecules with the first excited singlet state in polar solvents would not only reduce the energy of emission but would produce many vibrational quanta in the state formed immediately after emission. Since the number of vibrational levels in this state determines the shape of the emission spectrum, McClure and Edelman (6), give this as the reason for the good correlation between the lowering of the energy of the first excited state (which increases the wavelength emission maxima) and the increasingly broad fluorescence spectra.

Solvent Dependence of the Fluorescence Yield. The decrease in the quantum yield of these fluorophores with increasing solvent dielectric constant, in the primary alcohols, is inherently complex and several mechanisms involving both a decrease in the probability of radiative transitions and an increase in the probability of non-radiative transitions have been used to explain the solvent dependence of their fluorescence yield.

In their nanosecond time resolved spectrophotometric studies of ANS fluorescence Chakrabarti and Ware (9) have calculated the fluorescence lifetimes of the excited singlet state of ANS in various solvents of different polarities. The lifetime decreases with increasing dielectric constant of the solvent as the quantum yield of fluorescence decreases. Assuming fluorescence (with a rate constant k_r) and non-radiative processes (with a rate constant k_{NR}) are in simple competition with each other they

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calculated k_r: $k_r = Q_f/\tau_f$ and k_{NR} : $k_{NR}=(\tau_f)^{-1}-k_r$. Comparison of k_r and k_{NR} in solvents of different polarities allowed them to show that the quantum yield of fluorescence of ANS derives its dependence on the dielectric constant (or polarity) of the solvent both through a decrease in radiative and an increase in non-radiative processes. Thus ANS is less fluorescent in polar solvents both because non-radiative processes are enhanced and radiative processes diminished.

One mechanism of fluorescence quenching by increasing a non-radiative process is similar to the dipole orientation mechanism used in explaining the red shift in emission maxima with greater solvent polarity. More polar solvents show greater interactions with the larger dipole moment of the excited state molecule and lower the energy of the excited state. This reduction of the excited state energy increases the probability of internal conversion from the first excited singlet to the ground state. This would decrease the population of the excited state by enhancing radiationless processes and thus reduce fluorescence . This is also presumably why there is the corresponding decrease in fluorescence yield and decrease in the energy of emission as the polarity of structurally related solvents is increased.

The non-radiative process of intersystem crossing has also been used to explain the polarity dependence of these fluorophores fluorescence yields. Turner and Brand (7) have assumed that the dipole moment of the first excited triplet state is less than that of the first excited singlet state (a reasonable assumption in view of Murrell's calculations for aniline (11)). Since the energy of the first excited singlet state would be

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in more polar solvents, the energy difference between this state and triplet states would decrease. Kasha has shown that the energy difference between these states is a key factor in determining the rate of intersystem crossing (12). Intersystem crossing would therefore increase with increasing solvent polarity and fluorescence would be quenched by the enhancement of this non-radiative process.

Further support for the reduction of fluorescence yields by increased intersystem crossing in polar solvents is presented by Seliskar et al. (13). They showed an excellent correlation between the quantum yield and the square of the inverse of the singlet-triplet interval for N-phenyl-1-amino-naphthalene-7-sulfonate in many different solvents.

Non-radiative deactivation may also be possible through intramolecular motion (14). Several aminonaphthalene sulfonates which are fluorescent both in water and organic solvents will fluoresce only in organic solvents after their conversion to anilino naphthalene sulfonates (15). Other investigators have studied the relationship between molecular rigidity in solution and fluorescence efficiency and concluded that only planar molecules are fluorescent (16,17). Weber and Lawrence have suggested that the anilino naphthalene sulfonates fluoresce most efficiently when the anilino and naphthalene rings lie in the same plane. Intramolecular rotations or vibrations between the rings of TNS or ANS may cause internal losses of energy and serve to deactivate the excited state.

Increasing the viscosity of the solvent would slow such intramolecular motions and the quantum yield should increase in

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in more viscous media (14). Such increases in quantum yield with increasing viscosity have often been reported (6, 7, 9, 13). Both ANS and TNS show intense high energy emissions in viscous solvents such as glycerol.

Brand et al. (18) have studied the fluorescence properties of many naphthalene sulfonate derivatives. In one of their studies they show the similarity of the polarity effect on the fluorescence of TNS, 2,6 O-TNS and 2,6 M-TNS. All of these derivatives show low yields in water and high yields with a blue shifted emission maxima in non-polar solvents. The ortho derivative would give considerable hinderance to the rotation of the phenyl ring and all three compounds would have different orientations of the phenyl ring relative to the naphthalene ring. Because of the similarity of the polarity effects in these different derivatives it is unlikely that any particular orientation of the phenyl ring relative to the naphthalene ring is responsible for solvent effects on these fluorophores. Since the ortho derivative, where rotation of the phenyl ring is hindered, shows a similar fluorescence dependence on polarity it is unlikely that changes in intramolecular rotations are the mechanism of the fluorescence enhancements observed in more viscous solvents. However, changes in viscosity could alter the fluorescence by other means.

Chakrabarti and Ware, in their time resolved study of ANS fluorescence, have reported that the red shift in ANS emission which occurs in the nanosecond time range in polar solvents at room temperature and results in a reduced Q_f, does not develop under high viscosities. Thus highly viscous solvents seem to

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promote the high fluorescence yields and high energy (blue shifted) emission generally characteristic of much less polar solvents. In a highly viscous medium solvent reorientation about the excited state molecule may not be able to occur during the lifetime of the excited state and emission characteristic of a much less polar environment may occur, in polar solvents.

Fluorescent probe molecules such as TNS, ANS, or N-methyl, N-phenyl-2-amino-naphthalene-6-sulfonate, which do not fluoresce significantly in liquid water, show an intense high energy fluorescence in ice. Certainly the polarity has not changed upon freezing. However, the ability of the polar solvent molecules to reorient about the excited state of the fluorophore will be decreased in ice relative to liquid water. Seliskar et al. (13) have reported that N-methyl, N-phenyl-2-amino naphthalene-6-sulfonate in glycerol shows an increase in quantum yield and the energy of emission when (due to a temperature change) the solvent relaxation occured on a time scale larger than the lifetime of the dye molecule. These studies point out the key importance of solvent relaxation during the lifetime of the excited state in determining the fluorescence characteristics of such probe molecules. The main effect of increased solvent viscosity is to prevent solvent reorientation (even in polar solvents) during the lifetime of the excited state. This results in an increased fluorescence yield and energy of emission.

Thus in non-polar solvents, reorientation of solvent molecules does not occur because non-polar solvents do not interact strongly with the large dipole moment of the excited state

fluorophore. Fluorescence is intense and of high energy. In polar solvents, solvent reorientation can occur during the lifetime of the excited state and lower the fluorescence yield and energy. If such solvent reorientation is restricted (by viscosity or steric hinderance) in polar solvents, then the fluorophore will fluoresce as if in a non-polar medium.

Other investigators have implicated intramolecular charge transfer interactions as being responsible for the decrease in the radiative rate constant of these molecules in more polar solvents (9,13). They suggest such interactions occur between the lone pair electrons of nitrogen and the naphthalene ring electrons (a $\pi \rightarrow n_a$ transition). Chakrabarti and Ware have suggested that as the polarity of the solvent increases the contribution of the locally excited state decreases. This results in a decrease in the radiative transition probability and a decrease in fluorescence in more polar solvents. Thus in polar solvents such intramolecular charge-transfer interactions may contribute to the very low fluorescence yields.

Solvents other than the primary alcohols may have specific chemical quenching groups. In such solvents, even if they are rleatively non-polar, the fluorescence of ANS and TNS may be quite low. Acids such as acetic, propionic, and n-butyric, organic molecules with keto groups, amines, and pyridines, all blue shift the fluorescence of these fluorophores but give lower Q_f than those observed in the corresponding alcohols. McClure and Edelman (6) have suggested that the carboxyl groups of such acids quenches TNS fluorescence. They point out that solvents possessing delocalized electrons are effective quenching agents.

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This generalization is supported by Turner and Brand who have shown the quenching of ANS fluorescence with no corresponding shift in the wavelength emission maxima by solvents with unpaired electrons (7). Amines, pyrimidines, organic molecules with keto groups and bases such as diethylamine are all effective quenching agents for ANS. Such chemical quenching of fluorescence is common for many fluorophores and presumably results from the presence of electron donor or acceptor groups in solution which exhibit some intermolecular charge-transfer character towards the fluorophore.

D. Other Proposed Mechanisms of Fluorescence Change

Other researchers have offered different mechanisms to explain the very low fluorescence yields of these dyes in water. For reasons of completeness I will mention these mechanisms and point out why they could not play a major role in the solvent dependence of ANS and TNS fluorescence.

Stryer (5) has suggested that excited state proton transfer might be responsible for quenching ANS fluorescence in polar solvents. He reports a deuterium isotope effect with 5-amino-1-naphthalene sulfonate: (Q_f in D_2O)/ (Q_f in H_2O) = 3.04. That is, the quantum yield of fluorescence was enhanced in deuterium oxide. Stryer suggest that the proton donor group (NH_2) on the fluorophore was responsible for this effect. He argued that only the un-ionized species was fluorescent. Proton transfer from the NH_2 group to the solvent would result in an ionized species which was non-fluorescent. The rate of such proton transfer from donor to solvent would be slowed in D_2O and thus the fluorescence would be enhanced.

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Studies of the effect of pH on 1,7 ANS by Turner and Brand (7), indicate that the fluorescence yield, emission maxima and bandwidth are invariant over the pH range 1.5 to 12.0. The naphthylanilide ion forms only at very high pH, if it forms at all and therefore must be of little importance in terms of general solvent effects. These authors also studied the fluorescence of 2,6 M-ANS, in which the nitrogen is methylated and could not undergo a proton transfer reaction. The quantum yield of this compound exhibited the same strong polarity dependence. Again this suggest that proton transfer from the NH group of ANS in the excited state can not explain the polarity effects on its fluorescence.

Penzer has conducted fluorescence and proton magnetic resonance studies of ANS in methanol and water (19). He found that the benzene and napthylene rings are more nearly co-planar in methanol than in water. The NH proton in this case shows strong interaction with the sulfonate group probably through intramolecular hydrogen bonding and no proton exchange with the solvent occurs. In aqueous solvents the rings are not co-planar and no hydrogen bond formation between NH and the sulfonate group occurs. In this case a rapid exchange of the NH proton for solvent protons occurs. Penzer suggest that in aqueous solvents where NH-SO_3^- hydrogen bonding interactions do not occur, the nitrogen may hydrogen bond with water molecules. A molecular vibration of an N---H-O hydrogen bond between ANS and water would result in quenching of ANS fluorescence in aqueous solvents.

In organic solvents the NH group is not free to hydrogen bond with water because of its interaction with SO_3^- and its fluorescence yield is high. Viscous solvents could enhance

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fluorescence by decreasing vibrational deactivation of the excited state and by slowing solvent relaxation.

While the idea of intramolecular hydrogen bonding in ANS in organic solvents is very interesting and such hydrogen bonding probably does indeed exist, it cannot play a major role in altering the fluorescence parameters of ANS. This is because when the nitrogen of ANS is methylated an in 2,6-M-ANS neither this intramolecular hydrogen bonding nor intermolecular hydrogen bonding of ANS to water can occur and yet this compound shows similar fluorescence effects with polarity as ANS. Also 2,6 TNS 1,7 ANS and 1,5 ANS none of which can have intramolecular hydrogen bond formation all show similar fluorescence changes with polarity (7,18).

While intramolecular hydrogen bonding does not play a major role in the fluorescence characteristics of the anilino naphthalene sulfonates, it may be of importance in determining the fluorescence properties of the amino naphthalene sulfonates. Of the compounds 1,4 AmNS, 1,5 AmNS, 1,6 AmNS and 1,8 AmNS only 1,8 AmNS which can form intramolecular hydrogen bonds between the amino and the sulfonate group shows a very high quantum yield and blue shifted emission wavelength maxima in water.

Some very interesting results have come from X-ray crystallographic studies of TNS crystals by Camerman and Jensen (20). Two distinct types of TNS crystals were observed to form. The first was a hydrated crystal which had a broad weak, low energy emission similar to TNS in aqueous solution. When this crystal is dehydrated it cracks and an anhydrous crystal with a very intense, narrow, blue shifted emission (similar to TNS in organic

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The structure of TNS in this anhydrous crystal may be regarded as a planar naphthalene nucleus joined by a nitrogen to a planar tolyl group. The planes of their rings making an angle of 49.6 degrees to one another. The nitrogen atom is of nearly trigonal configuration with sp^2 hybridization and a p^2 lone pair. The overlap integral between the aromatic systems of the tolyl and naphthalene rings and the p^2 electrons of N is nearly as if all orbitals were parallel. The bond lengths between both C_{13}-N_{16} and C_{17}-N_{16} are considerably shorter than single C-N bonds. Such results strongly implies a delocalization of the p electrons of nitrogen into an extended π electron system consisting of the nitrogen atom and the benzene and naphthalene rings. There is thus evidence for electronic resonance stability over the entire molecule.

It is interesting, in this respect, that molecules which might interfere with the overall resonance of the molecule such as electron donors or acceptors might alter the emission properties of these fluorophores. Specific chemical bonding with the lone pair electrons on the nitrogen such as hydrogen bonding would be expected to alter the degree of intramolecular charge-transfer interaction and change the resonance throughout the molecule and its emission properties.

The X-ray crystallographic structure of ANS has also recently been determined by Cody and Hazel (21). In the crystal, ANS has been found to exist as two conformers called ANS(1) and ANS(2). The dihedral angles between the anilino and naphthalene rings are 63 and 53 degrees respectively. While the C-N-C angles

are nearly the same, 123.4 for ANS(1) and 127.3 degrees for ANS(2), the planes of the anilino and naphthalene rings make angles of 112/22^{o} in ANS(1) and -9/22^{o} in ANS(2), with the C-N-C planes. While they conducted no fluorescence studies on these crystals, these authors suggest that ANS(2) may represent the conformation of a fluorescent ANS molecule because the rings of the anilino and naphthalene groups are more nearly planar in this conformer. They found evidence for NH-SO_3^- intramolecular hydrogen bonding in both conformers.

From solution studies of "polarity probes" such as TNS and ANS some generalizations about factors which influence their fluorescence parameters may be made. The emissions of these probe molecules are extremely sensitive to the polarity of their environment. They show a large decrease in quantum yield and an increase in emission wavelength maxima and bandwidth with increasing solvent polarity in the absence of viscosity changes, specific steric hinderances and chemical quenching groups. The greater ability of polar solvent molecules to interact and reorient about the probe molecule during the lifetime of its excited state is responsible for their strong dependence of their fluorescence on polarity. In viscous mediums these fluorophores have a fluorescence similar to their emission in non-polar solvents. This may be partly because viscous solvents prevent degradation of electronic energy by rotational and vibrational motions in the molecule. However, the main factor in the viscosity effect seems to be a reduction in the ability of solvent molecules to reorient about the excited state fluorophore. Since factors such as viscosity and the presence of chemical

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quenching groups may alter the fluorescence of such "polarity probes" caution must be used when they are utilized to give a measure of the polarity of their environment.

III. APPLICATIONS OF FLUORESCENT PROBES TO BIOLOGICAL SYSTEMS

A. Polarity Probes and Proteins

The real value of fluorescence polarity probes lies in the extreme sensitivity of their fluorescence properties to the polarity of the medium around them. Weber and Laurence first discovered that ANS, while virtually non-fluorescent in water, became highly fluorescent upon binding to bovine serum albumin (BSA) (15). Apparently BSA has hydrophobic binding sites for ANS and when this probe is bound to these sites it experiences what is basically a hydrocarbon environment. Such hydrophobic binding sites would be composed of a crevice in the protein surface formed by non-polar amino acids such as leucine, valine, isoleucine and perhaps aromatic amino acids such as tryptophan, tyrosine and phenylalanine. In such an environment, the probe fluoresces intensely and at high energies as in a non-polar organic solvent.

The work of Stryer (4) on the binding of ANS to apomyoglobin has aided our understanding of the factors which influence ANS fluorescence. This study also serves to exemplify one of the ways in which such polarity probes may be used.

Stryer chose to study apomyoglobin (myoglobin without its heme group) because crystallographic studies had shown that it possesses a very non-polar heme binding site and it was expected to bind ANS at this site. ANS was shown to bind to a specific site on apomyoglobin with a dissociation constant on the order

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of 10^{-5}M. Addition of hemin resulted in the removal of the bound ANS from its apomyoglobin complex. This suggests that the ANS molecule was bound in or near the non-polar heme binding site on this protein.

Upon ANS binding to apomyoglobin the quantum yield of fluorescence increased from .004 to .98 and the emission maxima decreased from 515 to 454nm. Similar results were found for apohemoglobin which also possesses a very non-polar crevice for the heme group. These fluorescence changes of ANS provides an extremely sensitive means of monitoring the binding of hemin to these proteins and conformational changes that occur in these proteins and influence ANS binding.

Thus when a polarity probe is bound to a hydrophobic pocket of a protein its fluorescence may be greatly enhanced. Changes in its fluorescence properties may be used to monitor displacement of the bound probe from its binding site. Conformational changes which occur in the protein and alter probe binding or the environment of the bound probe may also be followed by such fluorescence changes.

The hydrophobic effect has been reviewed and beautifully described by Tanford (22). It may be summarized as the tendency of non-polar or hydrophobic molecules to aggregrate rather than disperse throughout an aqueous solution. Such "hydrophobic forces" play an important role in many protein-protein, protein-ligand and membrane-protein interactions. Many proteins are observed to have active sites or secondary binding sites which appear as crevices in the protein structure formed by hydrophobic amino acids. Such hydrophobic sites often exist as a part of the architecture of the protein structure which confers to

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proteins the high degree of specificity that they display in their interactions. Often such a site exist on the protein to facilitate a specific biological function. Hydrophobic forces may also lend a degree of stability to a certain three dimensional conformation of a protein. The conformational changes that occur about such hydrophobic sites are often important to protein function and these are specifically the type of protein conformation changes that one would wish to monitor.

Because of the hydrophobic nature of ANS and TNS (because of their aromatic rings) they are believed to often be directed to hydrophobic binding sites on proteins (4,6,23). When bound to such a site on a protein they fluoresce intensely and at high energies. Conformation changes around such sites which bind ANS or TNS may be monitored by changes in the fluorescence of these probe molecules. In this way many important structural changes in proteins have been easily monitored.

While these molecules are hydrophobic it should be realized that they are also charged molecules with a negatively charged sulfonate group. Hydrogen bonding could also be possible with the NH group of through the SO_3^- . Thus hydrogen bonding and ionic interactions of these molecules with proteins may also be important factors in their binding to proteins.

Some studies indicate that this charge does not play a major role in the binding of these probes to proteins. Gally(24) suggest that the aromatic nature of the ring structure is mainly responsible for the high affinity of these molecules for the hydrophobic sites and crevices of proteins.

Indications that electrostatic interactions are important

in the binding of TNS to proteins comes from studies of the structural requirements for binding and fluorescence enhancement of TNS by Beyer et al. (25). They have studied the binding of TNS (by rapid thin film dialysis and fluorescence) to naturally occuring cyclic peptide antibiotics: tyrocidines A, B, and C and gramicidin S-A and bacitracin A. TNS was found to bind to all the peptides except for succinyl-tyrocidine B. Fluorescence enhancements were only observed with three tyrocidines and these were the three peptides that could undergo self-association through hydrophobic interactions. They suggested that binding of TNS to these peptides was through ionic interactions of the negatively charged sulfonate group of TNS and a positively charged ornithine δ-amino group of the tyrocidines and gramicidin S-A. Succinyl-tyrocidine B does not bind TNS because the ornithine δ-amino group has been succinylated. Binding alone does not result in fluorescence enhancement. But fluorescence enhancement requires self-association which puts the aromatic ring system of TNS in a hydrophobic environment in the interior of the peptide aggregrate.

Binding may be enhanced by hydrophobic forces but it mainly results from ionic interaction with these peptides. First ionic interactions result in the binding of TNS to the peptides and then in those peptides which can associate, association results in the positioning of TNS in a hydrophobic environment and the observed fluorescence enhancement.

It is likely that both ionic and hydrophobic interactions play some role in the binding of ANS and TNS to various proteins. Other forces may also be involved. The role of each type of

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interaction in probe binding may vary with different proteins. However, only when the probe is bound to a hydrophobic site or bound such that nearby polar groups are restricted from interacting with the bound ANS during its excited state lifetime, would it be expected to fluoresce intensely and at high energies. In the latter case the probes intense fluorescence would result from what might be considered a high "microviscosity" in the probe binding site.

An indication that high energy intense fluorescence, like that observed in non-polar solvents, may occur from interactions that are not hydrophobic in nature has come from the studies of Seliskar and Brand (13). They have studied the interaction between the fluorescent probe N-methyl-N-phenyl-2-amino naphthalene 6-sulfonate and the cyclic sugar cycloheptamylose. This fluorophor bound with the sugar with a 1:1 stoichiometry and exhibited a large increase in fluorescence and a blue shift in its emission wavelength maxima upon binding.

The structure of this sugar is well nown and it has a cavity which has hydroxyl groups both on its inside and outside. The cavity is by no means hydrophobic. They suggest that the probe binds to this sugar (supposedly at this cavity) and upon binding its fluorescence is enhanced. The fluorescence enhancement, in this case, does not result from binding to a hydrophobic site since its binding site is very polar. Instead its binding is thought to restrict solvent reorientation and the probe then fluoresces as if it were in a non-polar environment. Thus in some cases probes may be bound at a polar site in a manner where the reorientation of polar molecules is restricted. Under such

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Time resolved emission spectra have supported the concept that the ability of polar solvent molecules to reorient about the excited state fluorophore determines its emission characteristics in that solvent (9, 10). Similar studies have been conducted with fluorescent dyes absorbed to biological compounds.

Brand and Gohlke (10) have shown red shifts in the emission spectrum of TNS bound to BSA in the 2 to 20 nanosecond time range. The effect of temperature on these shifts support the conclusion that these spectral shifts result from the nanosecond relaxation of polar groups around this bound dye during the lifetime of its excited state. Presumably the red shifts directly reflect the nanosecond motions of polar groups at the TNS binding site of BSA. Thorndill (26) has reported similar red shifts of TNS fluorescence when bound to several native and denatured proteins. Easter and Brand (27) have reproted these gradual time dependent red shifts in a TNS lecithin vesical system.

Easter et al (28) have conducted an even more extensive study of the time dependent spectra of the TNS-egg lecithin vesicle system. Their study is unique in that they display a three dimensional representation of the fluorescence intensity of vesicle bound TNS at 7 degrees C, as a function of both time in nanoseconds and wavenumber. In the time interval from 1 to 24 nanoseconds after excitation, the fluorescence intensity continuously decreases as the emission spectra is red shifted. They interpret their results in terms of nanosecond relaxations of polar molecules about the excited state TNS molecule. These relaxation processes serve not only to lower the energy of the

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excited state but also reduce the fluorescence intensity.

In aqueous solutions, solvents at room temperature reorient to rapidly to be observed on the nanosecond time scale. Thus these studies suggest that in these systems not only is the fluorophore surrounded by polar residues (or at least accessible to polar residues) but that these polar residues have a restricted motion relative to what we might expect in liquid solution.

These studies support the suggestion that the high energy intense fluorescence of polarity probes such as TNS or ANS can arise from a decrease in the reorientation of polar molecules about the excited state probe. This, in turn, may result from two sources: the removal of polar residues from the vicinity of the probe such as when it is withdrawn from aqueous solution and bound to a non-polar crevice in the protein or from a restricted ability of the polar molecules around the probe to reorient during the lifetime of its excited state.

Regardless of the nature of binding and fluorescence enhancement of these probes, they have been useful in monitoring a vast number of molecular interactions and conformation changes. Their usefulness lies in the extreme sensitivity of their fluorescence to their environment. However, it must be realized that an enhancement of high energy fluorescence upon probe binding does not indicate, a prior, that the probe is bound by hydrophobic forces to a non-polar crevice of the protein. It merely indicates that the probe is bound to the protein in a manner such that the reorientation of polar groups about the excited state of the fluorophore is less than solvent reorientation in aqueous solution. These probes could bind through electrostatic

or hydrogen bonding interactions and exhibit intense fluorescence even in a very polar environment.

Changes in ANS fluorescence has been used to monitor many different protein conformation changes and protein-protein, protein-membrane, protein-ligand interactions. The number of such studies is far to numerous to mention each study individually. Instead, I refer the reader to several recent reviews on the utility of fluorescent probes and their applications. Even these reviews, however, are not comprehensive of their use: See Fluorescence Spectroscopy of Proteins (29), Fluorescence Probes for Structure (30), Use of Fluorescence in Biophysics: some recent developments (31) and Fluorescent Protein Conjugates (32).

The polarity or hydrophobicity of the active site or other binding sites on proteins is of importance in the binding of substrates, cofactors and other ligands or macromolecules and in the actual catalytic process. Because of this some investigators have attempted to use fluorescence polarity probes to measure the actual polarity of binding sites on proteins.

Turner and Brand have used the N-aryl amino naphthalene sulfonates to determine the polarity of binding sites on several proteins (7). They have studied the fluorescence properties of 1,7 ANS in various solvents and found a close correlation of increasing fluorescence yield, decreasing emission maxima and bandwidth with decreasing solvent polarity (as measured by the empirical solvent polarity scale of Kosower). These investigators suggest that it is possible to use the fluorescence properties of such a fluorophore to estimate the polarity of its micro-environment without a knowledge of the actual chemical composition

of this environment. Since the quantum yield of these polarity probes is sensitive to more localized quenching reactions (more specific solvent effects) they suggest using the fluorescence emission maxima as an index of the polarity that the bound probe sees.

1,7 ANS, 1,5 ANS, 1,8 ANS and 2.6 TNS whose emission maxima are all a function of polarity (Z) were adsorbed to twenty proteins. Plots of each of their emission maxima as a function of Z for various solvents were constructed and used as a standard curve. The emission maxima of each probe, when bound to a protein, then corresponded to a particular Z value. This Z value was taken as the polarity of the probe binding site on that particular protein. Generalizations that they arrived at from these Z values were that the polarity of the chymotrypsinogen site(s) exceeds that of the chymotrypsin site(s) and that classes of enzymes such as the kinases and the dehydrogenases have characteristic binding site polarities. The calculated Z values from 1,8 ANS were consistently lower than those determined from the other three probe molecules which gave reasonably good agreement for the Z value of any single protein.

The value of such studies is doubtful. They attempt to characterize the polarity of a probe binding site without having any indication whether or not the probe is binding at one or more than one site on the protein. Also there was no evidence that the different probes bound to the same site(s) on any particular protein. In this sense the probe(s) may not be monitoring the polarity of an actual binding site on the protein but an "average polarity" of all the sites to which that probe

binds. Different probes could also be binding to different sites and reporting on their polarities. In the absence of a more specific knowledge concerning individual probe binding, such studies are of little value.

It is also possible that the emission maxima could be altered by microviscosity effects at its binding sites as well as polarity. The probe could be bound in a crevice of the protein structure that offered resistance to solvent reorientation about the excited state molecule or to a polar site with a high microviscosity or even to a site which favored a certain conformation of the bound probe molecule. Such effects could alter the energy of the excited state and result in anomalous Z values for the polarity of that binding site. Thus while polarity probes could sometimes be useful indicators of the actual polarity of a particular binding site on a protein, their use as such is hindered by the fact that all fluorescence parameters may be altered by characteristics of the probe binding site other than its polarity alone.

The real usefulness of such probes lies in the ability to follow conformational changes about the site where the probe is bound. Any change in conformation which alters the environment of the probe will be reflected as changes in its fluorescence properties. It is also highly desirable to determine the number of bound probe molecules on any protein, the affinity of that protein for the probe and the actual probe binding site in each conformational state of the protein. When this is done, we can tell to what degree the fluorescence changes we observe are the result of differences in the number of probe molecules bound

to a specific site on the protein or the result of enviromental changes about the bound probe at a specific site. The major shortcoming of most probe studies lies in the failure of many investigators to completely characterize probe binding in this manner. Although conformational changes can be followed by changes in probe fluorescence without such studies, this additional knowledge is invaluable in understanding what types of molecular changes may actually be taking place during a conformational change.

B. Polarity Probes and Membranes

The successful use of probes such as ANS and TNS to follow conformational changes in proteins encouraged membrane researchers to apply such techniques to the problems of membrane biophysics. Large increases in ANS fluorescence occur when this probe is added to suspensions of lipids or many natural membranes. Changes in ANS fluorescence have been used to detect different energy states of membranes, to follow conformational changes associated with the excitation of nerve axonal membranes, to detect phase transitions between the lipid crystalline and liquid state, to determine the polarity and/or microviscosity and mobility within the membrane, to follow the interaction and accessibility of many ligands such as proteins, ions and anesethetics with the membrane and to determine the electrical charge or potential of membranes. The real usefulness of ANS as a probe of membranes as in proteins is not in giving a real value for any of the membrane parameters, but in following changes in such parameters in membranes as they are perturbed, modified or undergo their biological functions. For an excellent review of

their use as such in membrane systems see The Application of Fluorescent Probes in Membrane Studies (33). Other reviews of the fluorescence approach to membrane studies include: The Design and Use of Fluorescence Probes for Membrane Studies (34), Can Fluorescent Probes Tell Us Anything About Membranes ?(35), Fluorescence Probes for Structure (30), Plasticity of Biological Membranes (36), Fluorescent Probes in Membrane Studies (37), The Use of Fluorescent Probes for the Study of Membranes (38).

While these reviews, particulary reference 33 should serve as a most complete guide for those interested in using ANS or other fluorescent probes to study membranes and membrane phenomena, a few generalizing remarks on ANS probing of membranes are called for.

ANS has been used very effectively to monitor many types of changes in both natural and artificial membranes. Its binding to membranes is observed to be both electrostatic and hydrophobic in nature. It is thought to be bound or partitioned between the lipid and water phase, probably penetrating the lipid to a limited extent and enjoying interactions with the polar head groups of phospholipids and peripherial membrane bound proteins. Ans does not perturb the native structure of membranes except at very high concentrations (generally greater than 10^{-2}M). At high concentrations it may disrupt the membrane. It is because of these properties that it has been so useful in following so many types of membrane interactions. Again, caution should be used in interpretations of the molecular mechanism of membrane-ANS fluorescence changes since such changes may result from factors other than altered polarity alone.

The use of fluorescent probe molecules to analyse the structural-functional relationships in proteins and membranes on a molecular level has already become a mahor tool in the attach on such problems. The extreme sensitivity of the fluorescent technique, our growing understanding of the theoretical basis for interpretation of fluorescence changes and the synthesis of new probe molecules whose fluorescence is sensitive to particular aspects of their environment assure the continued use and success of fluorescent probes in all types of searches for the molecular basis of biological phenomena.

IV. CHYMOTRYPSIN

A. A General Introduction

Chymotrypsin (CHT) belongs to a class of enzymes known as serine proteinases. It has a reactive serine residue (Ser 195) which is essential for its activity as a proteinase. As a proteinase, it functions to catalyze the hydrolysis of polypeptide substrates. In the small intestine of mammals, CHT (which is also an endopeptidase) preferentially hydrolyzes peptide bonds whose carbonyl groups are contributed by aromatic amino acids such as tryptophan, tyrosine, or phenylalanine. CHT is not highly specific, however, for it can cleave the peptide bonds contributed by leucine and methionine quite effectively and even other peptide bonds; at a greatly reduced rate.

The protein molecule itself has a molecular weight of 25,305 amu. It consist of three polypeptide chains held together by five disulfide bridges. The three chains A, B and C have 13, 131, and 97 amino acid residues, respectively. Its revised amino acid sequence has been determined by Blow (39).

The three dimensional structure of CHT has been determined by the X-ray crystallographic technique by two independent groups of investigators: Blow et al.(40,41) and Tulinsky et al. (42,43). This structure is discussed in detail by Blow (44,45) and by Vandlen (46). I will mention only the grosser aspects of this structure.

The protein is roughly spherical with dimensions of roughly 45x33x34 Å. The active site of the protein contains a hydrogen bonded network between serine 195, histidine 57, and asparagine 102. Two of these three residues lie on the surface of the molecule (His 57 and Ser 195) while Asp 102 is buried in the

interior of the protein and hydrogen bonded to the back of His 57.

This hydrogen bonded network between Asp 102-His 57-Ser 195, acts as a charge relay system. It conducts electrons from the buried carboxyl group of Asp 102, through a polarizable resonating system of the imidazole ring of His 57, to the oxygen of Ser 195 at the surface of the molecule. Thus electrons are transfered from the interior to hydrogen bonds on the exterior of the molecule. By this process the oxygen atom of the side chain of Ser 195 becomes very nucleophilic and may attack the carbon atom of the carbonyl group in a substrate (47). The maintainance of this charge relay system is of critical importance for chymotryptic activity.

Adjacent to the charge relay system, lies a well defined active site cleft which is approximately 7 Å deep in the protein structure. This pocket or cleft is occupied by the tosyl group in tosylated CHT and by substrate analogues (48,49). The formation of the active site cleft and the mechanism of catalysis will be discussed in more detail in my consideration of the structural differences between CHT and its zymogen CG.

Non-polar amino acid side chains are generally buried in the interior of the molecule, often as aggregrates within the protein structure (43,46). Polar residues are generally on the surface of the molecule, more exposed to solvent. There is only a small amount of alpha-helical structure in CHT. A somewhat distorted helix forms with residues 164-173 and an undistorted alpha-helix forms with residues 235-245, all of which lie in the C chain. Much more common with this protein is antiparallel pleated sheets, and larger regions of distorted sheets (45).

B. The Activation of Chymotrypsin

Enzymatically active α-CHT is derived from its inactive precursor or zymogen Chymotrypsinogen (CG). CG is converted to α-CHT by the tryptic and chymotryptic hydrolysis of four peptide bonds and the release of two dipeptides.

CG consist of a single polypeptide chain held together by five disulfide bonds. The main step in the activation process seems to be the tryptic cleavage of the CG chain between arginine 15 and isoleucine 16. This process alone produced π-CHT which although unstable, is enzymatically active (50,51). The subsequent autolytic action of CHT cleaves the leucine 13-serine 14 peptide bond and removes the dipeptide Ser 14-Arg 15 to produce δ-CHT (52,53). The dipeptide threonine 147-asparagine 148 is then clipped out by autolytic cleavages between tyrosine 146 and threonine 147 and between asparagine 148 and alanine 149 (53).

In the first unified activation scheme as proposed by Desnuelle (54), the final autolytic clipping of the Thr 147-Asp 148 dipeptide in δ-CHT was proposed to produce α-CHT. This was the suggested series of events for rapid activation mixtures, where the ratio of the CG/trypsin is relatively low (approximately 30). Under slow activation conditions where this ra id is high (around 10^4), Desnuelle points out that limited autolysis may occur first and produce the neo-chymotrypsinogens. The subsequent action of trypsin and further autolysis on these neo-chymotrypsinogens would then produce α-CHT. The formation of α-CHT from δ-CHT has recently been challegened by Wright et al. (55).

Examining the crystalline properties of α and γ chymotrypsins,

Wright and his co-workers have proposed that the rapid activation of CG produces γ-CHT as the immediate product of autolytic action on δ-CHT. The inter-conversion of γ and α-CHT, as described by Corey et al. (56) does not represent an important pathway for the formation of α-CHT. Instead α-CHT is believed to be produced mainly through the neo-chymotrypsinogens as in the slow activation mechanism of Desnuelle. Crystallographic studies of these chymotrypsins revealed no differences in amino acid sequence nor any major structural differences,although the crystalline properties of γ and α-CHT are quite different (40, 57,58).

The difficulty in deciding which of these activation schemes is correct lay in the apparent similarity of γ and α-CHT in solution. Two more recent approaches have sought to use physical differences between these two chymotrypsins to determine which activation scheme is actually responsible for the production of α-CHT.

Variability in the dimerization constants of these chymotrypsins have allowed Miller et al. (59) to differentiate between γ and α-CHT. They were able to prepare both γ and α-CHT from trypsin free δ-CHT. The yield of α-CHT was so small that its routine production by this pathway was concluded to be unlikely. They found trypsin free δ-CHT to be stable under slow activation conditions. Their results support the activation scheme of Wright et al. where α-CHT is formed from the neo-chymotrypsinogens instead of from δ-CHT.

In the process of their investigation, Miller et al. isolated and partically characterized a "new" form of CHT, which they

called k-CHT. It has isoleucine and threonine and probably cystine as amino terminal residues. k-CHT was produced from trypsin free δ-CHT and may be converted to both γ and α-CHT. They suggest that k-CHT fits into the activation scheme between δ and γ-CHT.

This k-CHT is probably the same enzyme as the α_1-CHT found before (60,61). k-CHT has been recently prepared, characterized and its kinetic properties studied (62). It is δ-CHT after the autolysis of the tyrosine 146-threonine 147 peptide bond but before autolysis of the asparagine 148-alanine 149 peptide bond. That is, the THR 147-Asn 148 dipeptide has not yet been removed. Because this derivative was first called α_1-CHT, I will refer to k-CHT as α_1-CHT.

The difficulty of these approaches to determining the steps in the activation of CG, is that in both the dimerization assays and in crystallization, with the subsequent comparison of crystal morphology, the enzymes are subjected to prolonged periods of exposure to solvent conditions which are different from the activation conditions. It has, in fact, been shown that both pH and $(NH_4)_2SO_4$, used in crystallization can influence the species of enzyme crystallized (56,60).

More recently Avery and Hopkins (63) have used the different rates of urea denaturation of the chymotrypsins in situ to identify the products of the rapid activation of CG. They found δ-CHT to be the first stable enzyme formed. The only autolytic product of δ-CHT had a rate of denaturation identical to that of α-CHT. In situ formation of γ-CHT was not detected. Their results, which are more convincing because of the in situ conditions used, support the original idea of Desnuelle; that

rapid activation of CG results in the formation of α-CHT through the autolysis of γ-CHT. Earlier studies of the denaturation rates of various chymotrypsins also support their findings (64).

Thus two pathways lead to the formation of α-CHT from CG. Both the "rapid" and "slow" activation pathways yield the final end-product, α-CHT, although through different intermediates. Under slow activation conditions, where trypsin concentration is low, chymotryptic activity may produce autolytically the various neo-chymotrypsinogens which are then cleaved by trypsin to form α-CHT. Under rapid activation conditions (high trypsin concentrations) trypsin cleavage of the Arg 15-Ile 16 peptide bond may occur first to produce π-CHT. Autolysis of π -CHT then yields δ-CHT. Further autolysis of δ-CHT yields the intermediate α_1-CHT. Additional autolysis produces γ-CHT which is structurally very similar and may isomerize to α-CHT. The general scheme of activation by both pathways is shown in Figure 5, from Wright et al. (55). Both pathways are probably utilized extracellularly to some extent to produce α-CHT. depending on the concentration of trypsin.

C. Structural Changes with Activation

Both chemical and physical studies have shown that the enzymatic cleavage of a single peptide bond between Arg 15 and Ile 16 is sufficient to convert the zymogen to an active enzyme (65,66,67,68). The fact that such comparatively simple chemical changes are involved in the trypsic activation of CG to CHT, raises some serious questions concerning what types of physical and chemical changes occur to confer activity to this enzyme:

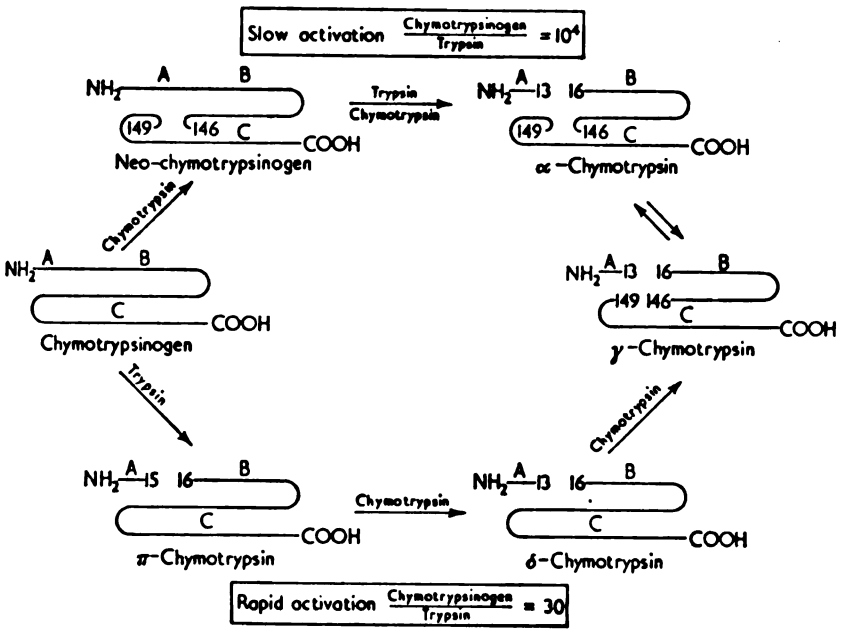

Figure 5 . The formation of the various forms of CHT (55).

1. Does the catalytic site already exist in the zymogen only to be unmasked by the primary tryptic cleavage ?
2. How is enzymatic activity related to the chemical changes involved in activation ?
3. Do conformational changes occur as a result of the primary hydrolysis over only a part or over the entire protein molecule, to create a specific enzymatically active configuration (69).

Such questions can only be answered through a more complete understanding of the structural differences between the zymogen and active enzyme. Several studies provide direct evidence that activation is paralled by important conformational changes.

The ultraviolet absorption spectra of the aromatic amino acids of proteins is known to be very sensitive to the structural changes in the proteins in which they reside. Chervenka (70) has shown that changes in the ultraviolet absorption of CG occur with its activation. Difference absorption spectroscopy shows a clear decrease in absorption near 287 nm upon tryptic activation of CG. This decrease in absorption parallels the rate of increase in chymotryptic activity. CHT itself is responsible for the autolytic conversion of π to δ-CHT and such autolysis is inhibited by β-phenyl proprionate, competitive inhibitors of chymotrypsin catalysis and by Ca^{++} ions (71,72). Since similar absorption changes occur in the presence and absence of these chemicals, Chervenka suggest these absorption changes near 280-290 nm may result from conformation changes around tyrosine or tryptophyl residues in a small segment of the protein molecule. His results provide support for the conclusion that conformational changes occur as an inseperable part of the

activation process.

Protein conformational changes are very often accompanied by changes in the circular dichroic (CD) and optical rotary dispersion (ORD) of the protein. Using specific rotation data on the conversion, Neurath et al. (73) have shown that changes in optical rotation parallel the occurance of enzymatic activity. They concluded that the higher specific levorotation of CG relative to CHT and its relative insensitivity to changes in pH and concentration, suggest that the zymogen is less coiled and more rigid that the active enzyme. Presumably, the primary tryptic cleavage allows the more extensive folding to occur.

Other investigators using ORD have concluded that CHT has from twice as much to only slightly higher helical content than CG (74,75). Raval et al. (76) on the other hand point out that in spite of the differences in ORD patterns of these proteins, they have very nearly the same helical content. They reached this conclusion on the basis of the depth of the 233 nm trough, b_o (the dispersion parameter) and absorption in the deep ultraviolet. Their work is supported by studies which show that the ORD changes in CHT, which were once thought to measure large structural changes in the protein are probably due to very local perturbations in specific chromophoric groups (77). These authors have also shown that large changes in folding (in the conversion of CG to CHT) may occur without significant changes in ORD. The confusion in interpretation of such ORD data is due to additional cotton effects which are not associated with helix or random coil conformation of peptides. These studies point out the difficulty of the analysis of ORD data

in terms of absolute structure. Even with complete ORD, conclusions on the degree of folding and changes in helical content are not available from ORD alone.

The main conclusions from ORD studies is the fact that the conversion can be followed by changes in the optical rotary power of the protein and the suggestion that activation results in an increase in folding of the protein structure. This latter finding is supported by the work of Biltonen and Lumry (78) who have studied Brandts' thermodynamic analysis of the transition of CG to CHT (79,80).

Many physical and chemical studies have also shown that CG does not bind substrates and substrate analogues as effectively as CHT. This suggest that CG has no active site cleft or specificity pocket. These studies have been supported by the X-ray analysis of CG by Freer et al. (81).

A broader understanding of the molecular events occuring with zymogen activation have come from a comparison of the three dimensional X-ray structure of CG and CHT. The preliminary comparison of these structures by Freer et al. reveals several important structural features. Although there are numerous localized differences in conformation, the overall folding of the polypeptide chain is similar in zymogen and active enzyme. Upon the tryptic cleavage of the Arg 15-Ile 16 peptide bond in CG, the main chain rotates 180 degrees about an axis defined by the α-carbon of residues 19 and 21. In this process the α-amino group of Ile 16 approaches the buried side chain carboxyl of Asp 194 to form the internal ion pair previously shown to exist in CHT by Matthews et al. (40). During ion pair formation, the

formerly exposed side chains of Ile 16 and Val 17 are buried. In CG, Asp 194 is also buried with its side chain hydrogen bonded to the side chain of His 40. Activation results in a rotation of 180 degrees about the carboxyl carbon to carbon bond of Asp 194. This shifts the side chain of Asp 194 from His 40 into the vicinity of the new position of Ile 16. This rotation causes large movements of Met 192.

The specific active site cavity near Ser 195 is not formed in the zymogen. A conformational change in the backbone and the shift of Met 192 from its completely buried position to the surface of the molecule serves to complete the active site cavity found in CHT. The amino acid residues nearby Met 192 which are folded into the CG molecule, move out to form the top of the hydrophobic binding pocket and the position they occupied in CG is taken by the Ile 16- Asp 194 ion pair upon zymogen activation. This finding was not surprising since the tryptic cleavage freeing Ile 16 is known to result in enzymatic activity and Oppenheimer et al. (50) have shown that an ion pair involving Ile 16 was essential for CHT hydrolysis of specific substrates.

The hydrogen bonded network linking Asp 102, His 57, and Ser 195 which had previously been shown to form a charge relay system at the active site (29), is preformed in CG. It was rather unexpected that the same charge relay system exists in the zymogen. Because of its known importance in the mechanism of CHT hydrolysis of substrates it was expected to be disrupted in the catalytically inactive CG. Although this preliminary comparison of the crystalline structure was imformative, the observed differences did not seem to explain sufficiently the

large changes in catalytic activity which occur upon activation. Freer et al. have suggested that small changes in the side chains of His 57, Ser 214 and Ile 16 might be responsible for the "turning on" of enzymatic activity.

Recently a more detailed study of the differences of the crystal structure of zymogen and enzyme and a comparison of their known chemical differences has lead to a more comprehensive understanding of the properties of CG and its activation to CHT. In this more detailed comparison, Wright (82) has supported the changes found by Freer et al. and reveals several additional structural changes which may account more fully for the high catalytic activity upon zymogen activation. He list all of the significant structural differences between these two molecules (changes greater than 1.2 Å which extend through three or more residues). He further points out the changes in hydrogen bonding which accompany such structural changes. While changes in the main chain hydrogen bonding are few, nearly half of the hydrogen bonds formed with side chains are different in the two structures. Wright confirms the finding that the residues of the charge relay system are nearly identical in both proteins, however, there are changes in the environment of these residues.

The mechanism of chymotryptic hydrolysis has been worked out in great detail from both solvent and crystallographic studies and the mechanism proposed by Henderson et al. (83) is supported by a very large amount of evidence. This subject has been extensively reviewed (84,85,86,87). This proposed mechanism of CHT action is shown in schematic detail in Figure 6, taken from reference 82.

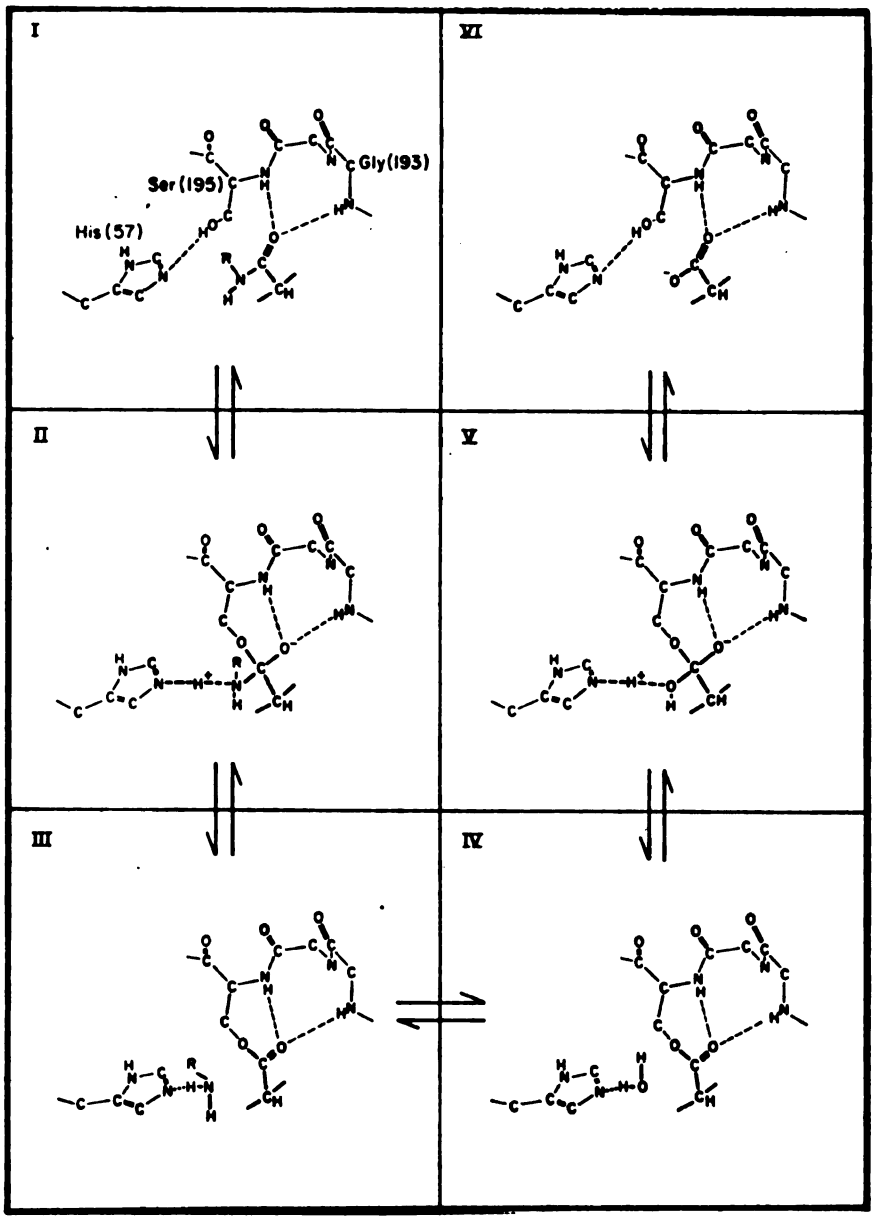

Figure 6. The proposed mechanism of Chymotrypsin-catalyzed hydrolysis.

In accordance with the mechanism of CHT action, Wright points out nine specific substrate interactions which are necessary for such hydrolysis to occur:

1. The binding of side chain in the primary specificity pocket (49).

2. A hydrogen bond between the acylamido group of the substrate and the carbonyl oxygen of Ser 214 (49).

3. A hydrogen bond from the $N_{(\varepsilon_2)}$ of His 57 to the leaving group of the substrate (49,88).

4. and 5. Two hydrogen bonds, one from the oxygen of the substrate to the amido-NH of Gly 193 and the other from this same oxygen to the amido-NH of Ser 195 (89).

6. The covalent bond between O of Ser 195 and the carbonyl carbon of the substrate (89).

7. and 8. Two hydrogen bonds from Gly 216 of the enzyme to the complementary hydrogen-bonding atoms of the peptide group in a residue of the substrate (90).

9. The interaction of the substrate leaving group with the side chain of Met 192 and Cys 42 of the enzyme (91).

These enzyme-substrate interactions decrease the entropy of the bound substrate, increasing the liklihood that it will lie in an active conformation and thus reduce the free energy of activation for the hydrolysis reaction.

Wright points out that four of these nine interactions cannot occur in CG as they do in CHT. These are; binding in the specificity pocket, the Gly 193-NH hydrogen bond, the hydrogen bond from the substrate to Gly 216, and the interaction of a polypeptide substrate leaving group with Met 192. The inability

of CG to fulfill these enzyme-substrate interactions is given as the reason for the 10^5 to 10^6 decrease in activity towards specific substrates that CG sees relative to CHT.

Thus even though the charge relay system is preformed in the zymogen, it is in a different environment and does not have the ability to bind substrates in the precise orientation necessary for efficient catalysis. This considered with the other environmental changes in the active site, especially the absence of Gly 193 (as a hydrogen bond donor), satisfactorily explains why CG is inactive relative to its activation product CHT. Such chemical and crystallographic studies have proven invaluable in determining the actual structural changes which occur upon zymogen activation to active enzyme and how such structural changes so dramatically influence function.

D. The pH Dependence of Chymotrypsin Conformation

The conformation of a polypeptide chain of a protein is determined by its primary amino acid sequence and by the size, shape, and polarity of its amino acids. The nature of the amino acids and their order in the polypeptide chain determine the type of interactions that they may undergo to stabilize a particular three dimensional structure. Changes in the ionization state of a particular amino acid may also alter its ability to be involved in specific types of interactions. Interactions such as van der Waal's , hydrogen-bonding, dipole-dipole, and electrostatei, are of special importance in maintaining any particular conformation of a protein.

The peptide bond is the only covalent linkage between amino acids in the backbone of the polypeptide chain. The pH dependence

of the conformation of a polypeptide is determined by the NH_2 amino terminal, the COOH carboxyl terminal and those R groups which may ionize. The pK_a's for the ionization of the free amino acids may be found in most good biochemistry texts or reference 92. The pK of a particular amino acid may be quite different in a protein because its electrostatic environment may be altered by the interactions that occur in the protein (93). Since the state of ionization can influence the types and strengths of interactions that serve to form and stabilize protein structure, it is not surprising that conformational changes so often occur with pH changes. Chymotrypsin is a good example of how even subtle pH changes can dramatically alter both conformation and activity.

The dependence of CHT conformation on pH is complex but some of its most salient features should be mentioned. One of the most interesting of these is the electrostatic interaction or salt bridge formed between the α-amino group of isoleucine 16 and the β-carboxyl group of aspartic acid 194. This salt bridge as revealed by X-ray crystallography, is buried inside the protein and rather shielded from solvent (40,41). At neutral pH the salt bridge is intact and the enzyme is active. At low pH (pK approximately 3.0) the salt bridge is disrupted by the protonation of the β-carboxyl group of Asp 194 (94). At high pH (with a pK of approximately 8.0 for the inactivation of the enzyme by this process) the salt bridge is disrupted by the deprotonation of the α-amino group of Ile 16 (95).

Large and similar changes in protein fluorescence occur when the enzyme is inactivated by either acid or alkaline pH (94).

This suggests that the same phenomenon, the disruption of the salt bridge is occuring to inactivate the enzyme at both pH extremes.

The crystal structure of CHT at pH 8.7 shows that the salt bridge between Ile 16 and Asp 194 is disrupted by the deprotonation of the α-amino group of Ile 16. These residues then move away from one another and a major reorganization of nearby residues is observed (96). Thus the formation and maintaince of the Ile 16-Asp 194 salt bridge is necessary for activity. When this bridge is not formed as in CG or not maintained as in the pH-extremes of CHT no specific substrate binding nor activity is observed.

In CG the α-amino group of Ile 16 is in a peptide bond with Arg 15 and cannot interact with Asp 194. The main event in activation appears to be the tryptic cleavage of the Arg 15-Ile 16 peptide bond which frees the α-amino group of Ile 16, allowing it to form a salt bridge with Asp 194. The resultant movement of the peptide chains in the formation of this salt bridge forms a part of the active site cleft of CHT and allows the specific binding of substrates (81).

It has been suggested that CG has essentially the same structure as the high pH form of CHT, since in each the Ile 16-Asp 194 salt bridge is not formed. The disruption of this salt bridge by extremes of pH in CHT presumably induces conformational changes that parallel the structural changes between CHT and CG upon activation (41). When this salt bridge is disrupted (the charge of Ile 16 is altered by deprotonation or through chemical modification) the enzyme is in a conformation which is similar

to that of CG in terms of its circular dichroism, optical rotary dispersion and its activity towards specific substrates (50,94, 97,98,99,100,101,102). This proposal is also supported by the similarity in the conformational changes of CHT in going from pH 3.6 to 8.7 and in CG upon its conversion to CHT. Mavridis has shown that many of the high pH changes in CHT that occur with disruption of the Ile 16-Asp 194 ion pair are qualitatively similar to the conformational changes that occur with zymogen activation (96).

There exist a second internal ion pair in this protein. It is a salt bridge between the carboxyl of Asp 102 and the ring of Histidine 57. At low pH (5 or 6) this ion pair is believed to be uncharged. It gains a negative charge with a pK of approximately 6.7. Johnson and Knowles (103) have suggested that such a salt bridge and negative charge in the active site could explain the pH dependence of the binding of anionic inhibitors to CHT.

The pH dependence of CHT catalytic activity implies that a group in the active site with a pK of 6.7 inactivates the enzyme. This group has been identified as the imidazolium group of His 57. Since the proton of N_{ϵ} of His 57 is buried in the interior of the protein and stabilized by hydrogen bonding to Asp 102, Blow et al. ( ) have suggested that it is the N_{ϵ_2} of His 57 that is deprotonated with a pK of 6.7. This deprotonation would disrupt the hydrogen bond between His 57 and Ser 195. However, the serine hydroxyl group can freely rotate and without any perturbation of structure assume a position favorable for hydrogen bonding to $N_{\epsilon 2}$ of His 57. This apparently sets the

stage for biological activity. It is interesting to note that even though the nature of the hydrogen bonding has changed there still exist a hydrogen bonded network from Asp 102 to His 57 and from His 57 to Ser 195.

At pH 8.7 where the His 57 would have been deprotonated the crystal structure shows a small shift of Ser 195 away from the plane of the imidazole ring of His 57, with no change in the position of the histidine (96). This is fully consistent with the disruption of one hydrogen bond and the formation of a new hydrogen bond between His 57 and Ser 195, with little actual change in the geometry of the active site. Such a hydrogen bonded network is also the basis of the mechanism of CHT catalysis that is now generally accepted.

Considering these two internal ion pairs we can account for the pH dependence of CHT activity as shown in Figure 7, for the hydrolysis of N-acetyl-L-tryptophan amide (104). When His 57 is deprotonated near pH 6.7, activity begins and becomes maximal at 7.5 to 8.5 depending on the experimental conditions. At higher pH the activity is rapidly reduced due to the disruption of the Ile 16-Asp 194 salt bridge by deprotonation of the α-amino group of Ile 16.

The pH induced conformational changes in CHT have been followed by a variety of different techniques designed to monitor specific changes in protein structure. None of these other methods even approach the resolution provided by X-ray crystallography. The approach to structural changes as a function of pH, determined by high resolution X-ray crystallography is particularly meaningful. The conclusions reached by other

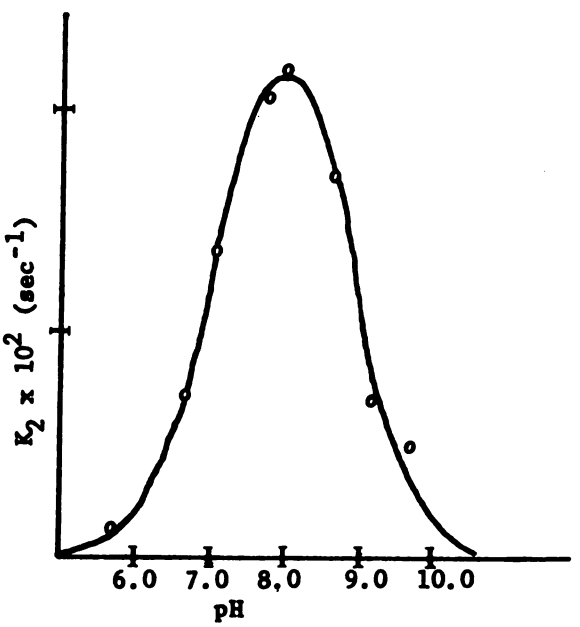

Figure 7. The CHT - catalyzed hydrolysis of N-acetyl-L-tryptophan amide as a function of pH (104).

experimental approaches must be compatible with the results of crystallographic studies or exhibit differences that are explainable in terms of the differences between the protein in solution and in the crystal.

It is interesting, in this sense to see that many structural changes which had been detected by other physical approaches have been confirmed by X-ray analysis. For example, the molecular basis for the pH dependence of CHT activity, dimerization, intrinsic fluorescence, CD and ORD can be explained in terms of the structural changes that occur as a function of pH as revealed by X-ray crystallography (83,87,96,105,106).

From X-ray studies which reveal the structural changes induced by changing pH from 3.6 to 8.7, it is clear that dramatic rearrangements occur in protein structure with pH. While large conformation changes occur across the molecule with the disruption of the single Ile 16-Asp 194 ion pair, smaller and more localized changes occur with the disruption of other interactions. i.e. the hydrogen bonding of His 57 to Ser 195. Although more localized, these latter type of changes may seriously effect chymotryptic activity.

Many such localized changes occur in CHT as the pH is raised in this range. It has been shown that the surface charge may change as much as ten units as a result of the deprotonation of basic groups on the protein (96). In addition to the effects of altering the surface charge of the protein alone, changes in the interactions of solvent, ions, and other molecules with ionizable polar residues can have very large effects on protein structure and activity. Such changes can only be seen in all of

their detail by high resolution crystallography.

The major changes which occur with such a pH change in CHT are presented in recent publications (105, 106) and these changes and more detailed smaller changes are presented in a recent dissertation (96). Numerous changes in CHT structure occur with changing pH. While some are very localized others are quite extensive. I have mentioned only some of the pH induced changes which regulate CHT activity, for a more detailed presentation and discussion the reader should consult the original treatments.

V. SOLUTION STUDIES OF CHYMOTRYPSIN-ANS

A. The pH Effect

We have found the fluorescent probe molecule, ANS, useful in monitoring conformational changes in chymotrypsin, chymotrypsin derivatives and chymotrypsinogen. The most pronounced effect we have observed is the pH dependence of ANS fluorescence in solutions of these proteins.

Below pH 4.0 CHT-ANS solutions exhibit an intense blue-green fluorescence. As the pH is raised the intensity of this fluorescence drops dramatically and a very weak yellow emission is observed. These intensity changes can be clearly seen in Figure 8 which compares the relative intensities of a CHT-ANS solution (10^{-4}M CHT and 2×10^{-5}M ANS) at pH 3.6 and pH 7.0. Successive red shifts to a lower energy emission also occur with increasing pH. This is shown in Figure 9. Thus as the pH of CHT-ANS solutions is raised from acid to neutral or alkaline pH the fluorescence intensity is decreased as much as 50 fold and the emission maxima is red shifted more than

20 nm. The fluorescence of ANS in solution alone is insensitive to pH changes in this range. The pH profile of CHT-ANS fluorescence intensity is shown in Figure 10.

Chymotrypsinogen also binds ANS and the pH profile of CG-ANS fluorescence intensity is also shown in Figure 10. Although the fluorescence intensity in CG-ANS solutions is strongly pH dependent, the emission maxima remains near 475 nm both at low and high pH. While the fluorescence intensity of CHT-ANS and CG-ANS solutions is very similar at low pH, they exhibit distinct differences in the changes of their intensity and energy of their fluorescence with pH.

ANS is virtually non-fluorescent in water and yet fluoresces intensely when in a non-polar medium and when bound to many proteins. Thus we know that ANS is bound to CHT (at least at low pH) because of its intense fluorescence in CHT solutions. The question we then want to answer is whether or not ANS binds to CHT at its active site. We initially felt that the ANS binding site was distinct from the active site of CHT since an active site cleft exist only in CHT and yet both CHT and CG bind ANS and fluoresce intensely at low pH. A variety of other evidence support this conclusion.

B. <u>The ANS Binding Site as Distinct from the Active Site</u>

CHT-ANS complexes, in which the active site is blocked with toluene sulfonyl fluoride, or the presence of 0.01 M β-phenyl proprionate (a competitive inhibitor of CHT activity) both show the same fluorescence-pH profile as native CHT in ANS solutions. See Figure 10 for the tosylated CHT-ANS pH profile. These studies show that when the active site of CHT is blocked

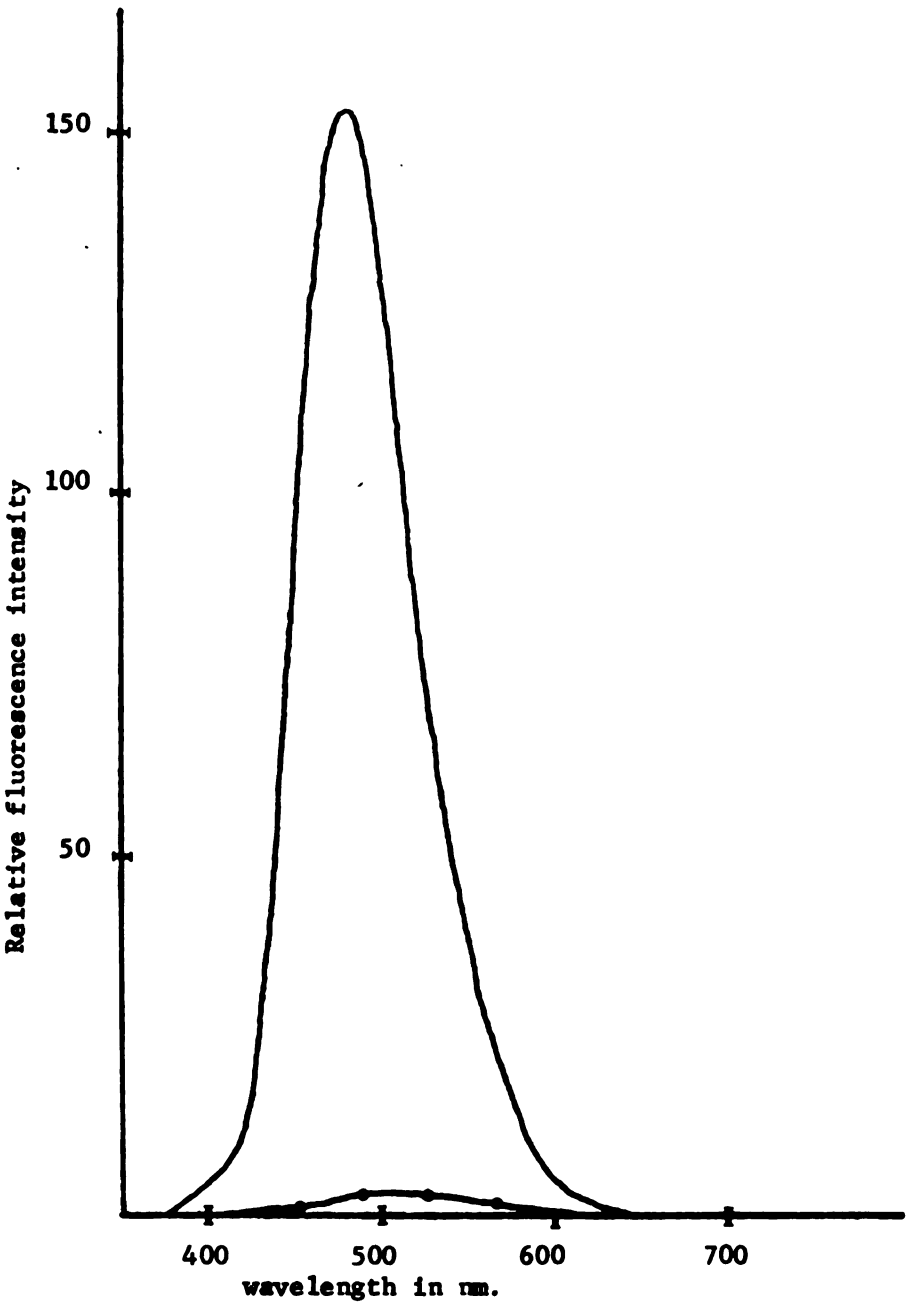

Figure 8 . The fluorescence spectra of CHT-ANS at pH 3.6 (——) and 7.0 (—•—).

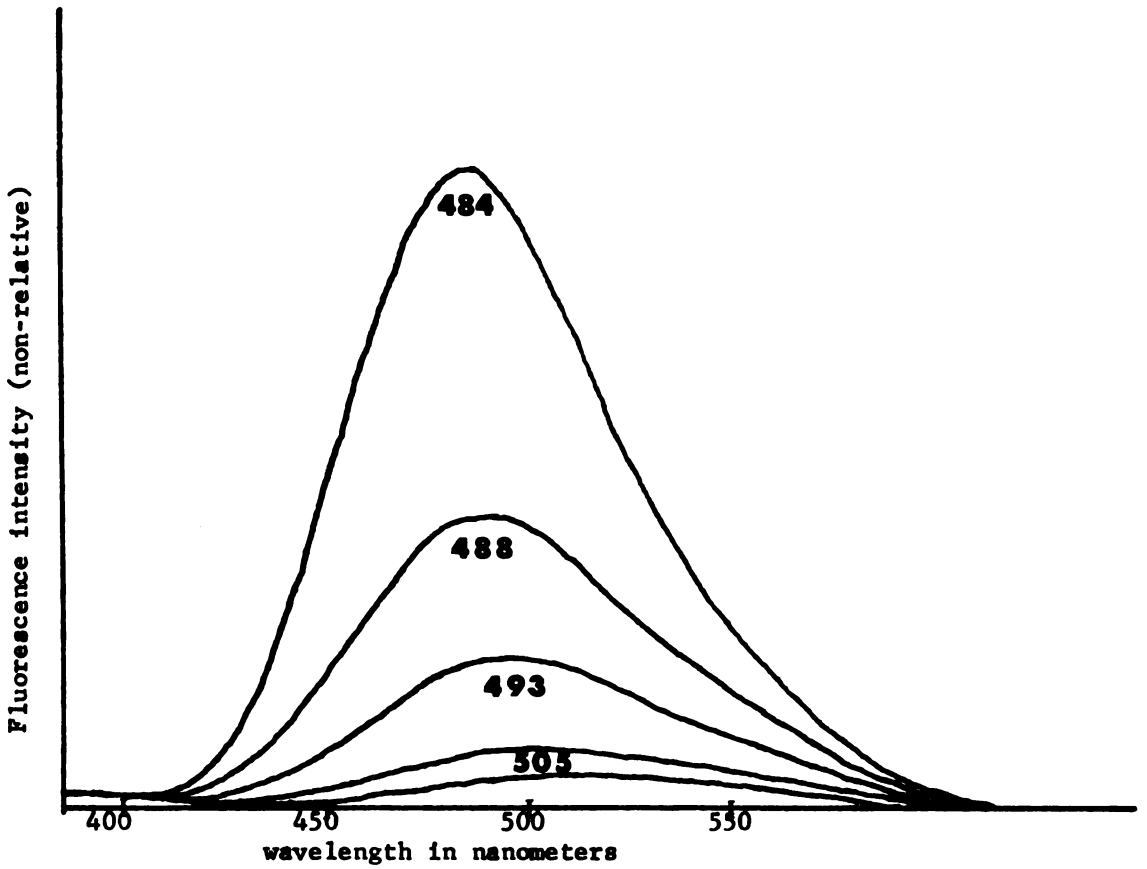

Figure 9. The successive red shifts of the emission maxima of CHT-ANS at pH 2.4, 3.6, 4.75, 7.0, and 8.0

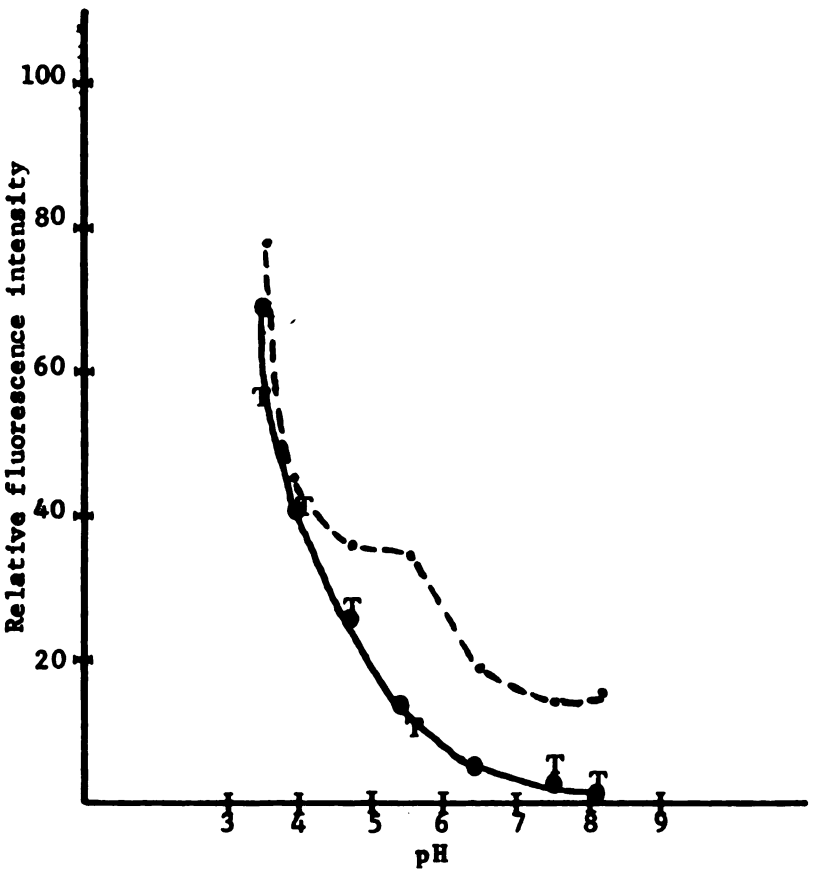

Figure 10. The pH dependence of protein-ANS fluorescence. CHT (●), tosyl-CHT (T), CG (•).

the fluorescence and thus binding of ANS is not affected.

If ANS were bound to the active site of CHT it should inhibit catalysis. A standard assay for CHT activity involves the enzymatic hydrolysis of acetyl tyrosine ethyl ester (ATEE). This hydrolysis is a measure of chymotryptic activity and may be followed by the decrease in absorption of ATEE at 237 nm(107). The hydrolysis of ATEE by CHT was monitored by this decrease in absorption and found to be the same in the absence and in the presence of concentrations of ANS even as high at $4x10^{-4}M$.

Proflavin is known to bind to the active site of CHT and to inhibit activity. Upon binding, its absorption maxima is shifted from 444 to 465 nm. (108,109). The amount of bound proflavin can be followed by changes in its absorption maxima. Using this technique, the binding constant for many substrates and substrate analogues to CHT have been determined (110). No change in the absorption maxima was observed with the addition of concentrations of ANS as high as $2x10^{-4}M$. This suggest that ANS neither competitively nor non-competitively inhibits the binding of proflavin to the active site of CHT. Thus a wide variety of solution studies all suggest that ANS does not bind to the active site of CHT nor does it affect catalytic activity or the binding of competitive inhibitors to this enzyme.

C. <u>The Affinity of CHT and CG for ANS at pH 3.6 and 6.4</u>

After determining that ANS bound to a site other than the active site of CHT it was of interest to determine the equilibrium binding constants of ANS to CHT and CG at both low and high pH. Figure 11 shows the results of titrations of protein ($5x10^{-5}M$) with ANS (10^{-5} to $2.5x10^{-4}M$). A plot of

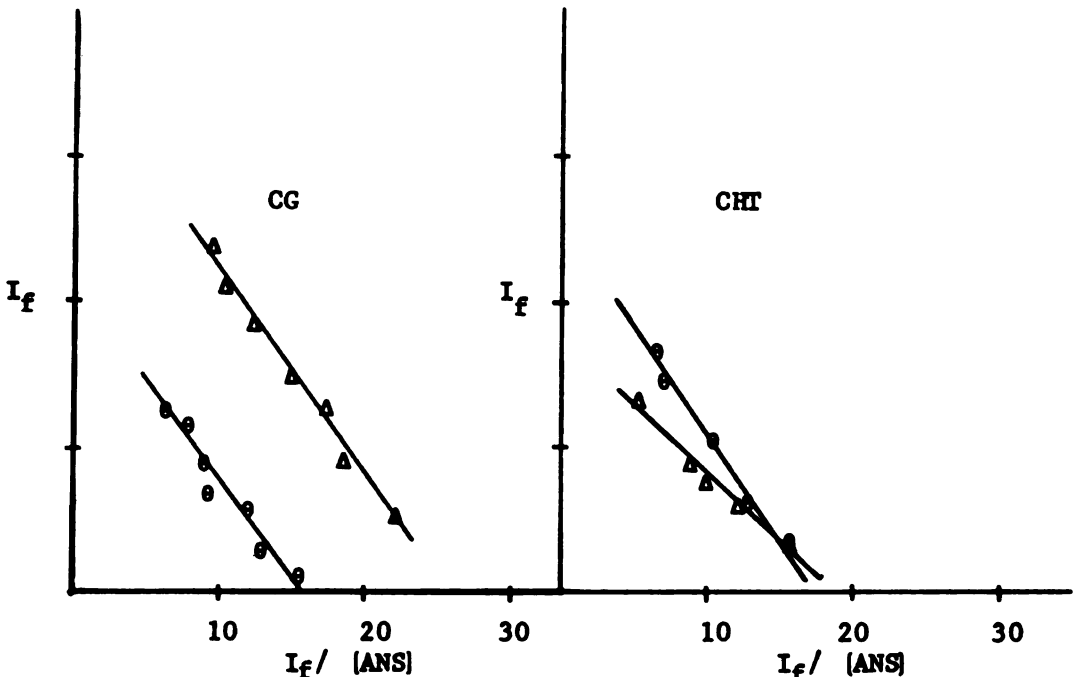

Figure 11. Titrations of CG and CHT with ANS, pH 3.58 (θ) and pH 6.38 (Δ).

the intensity of fluorescence (I_f) versus I_f/ [ANS] gave straight line plots with intercepts on the ordinate equal to the maximum fluorescence of the protein-ANS complex and a slope equal to $-K_d$, the dissociation constant of the complex. K_d for the CG complex was 1.3×10^{-4} and 1.4×10^{-4} M for pH 3.6 and 6.4 respectively. K_d for the CHT complex was 1.4×10^{-4}M and 1.0×10^{-4} M for pH 3.6 and 6.4 respectively. The dissociation constants only varied between 1.0×10^{-4} and 1.4×10^{-4} for CHT-ANS and CG-ANS at pH 3.6 and 6.4. Such a small variation in equilibrium binding constants of both proteins for ANS at both pH values suggest that ANS binds to CHT and CG with equal affinity and in equal amounts regardless of pH. Thus the fluorescence changes observed with pH do not result from changes in the amount of ANS bound but rather from changes in the environment of the bound ANS.

Presumably under low pH conditions, ANS is bound to these proteins in a manner that restricts solvent reorientation about the excited state fluorophore. At higher pH, ANS is still bound to the protein with a similar affinity but in a manner where solvent reorientation about the excited state ANS may occur more readily. Thus at low pH, intense high energy fluorescence is observed relative to protein-ANS solutions at a higher pH. This change in the ability of solvent cage relaxation to occur could result from pH induced changes in the conformation of CHT around the ANS binding site.

D. The Number of ANS Binding Sites on CHT

Several Classical types of analysis, involving titrations of protein with ligand or ligand with protein, can give

quantitative results on the number and affinity of bound ligands (111). For such analysis it is necessary to determine the fraction of bound ligand. For ligands like ANS, which fluoresce very intensely when bound and are virtually non-fluorescent in aqueous solution alone, the fraction of bound ligand X can be written as $X=F_p/F_{max}$. Where F_p is the fluorescence intensity of ANS in the presence of protein and F_{max} is the fluorescence intensity at protein concentrations where all the dye is bound. The use of this formula requires that the fluorescence intensity is a linear function of ligand or protein concentration.

Titrations of fixed protein ($5x10^{-5}M$) with increasing concentrations of dye (5.0 to $70.0x10^{-6}M$) and fixed dye with increasing concentrations of protein were conducted at pH 3.6 in 0.067 M phosphate buffer. In this range the fluorescence intensity increased linearly as a function of protein or dye concentration. A plot of the reciprocial of fluorescence intensity versus the reciprocial of protein concentration gave a straight line plot intercepting the ordinate at F_{max}.

If D_o is the total dye concentration, then XD_o is the amount of bound dye and $(1-X)D_o$ is the concentration of free dye. Letting $V = XD_o$ / Protein and defining $C = (1-X)D_o$, a plot of 1/V versus 1/C is shown in Figure 12. The intercept on the 1/V axis is equal to 1/N and its slope is equal to $1/K_aN$. Here N is the number of ANS molecules bound per protein and K_a is the classical first order association constant. This plot indicates that approximately 1.1 ANS molecules are bound per chymotrypsin, with an affinity of approximately $2.6 \times 10^4 M^{-1}$.

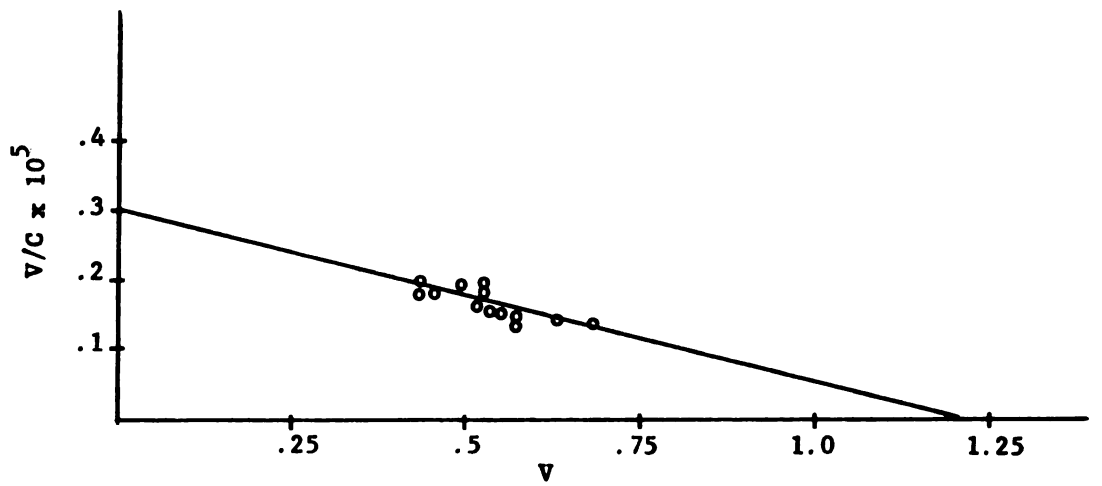

Figure 13. A Scatchard plot of ANS binding to CHT .

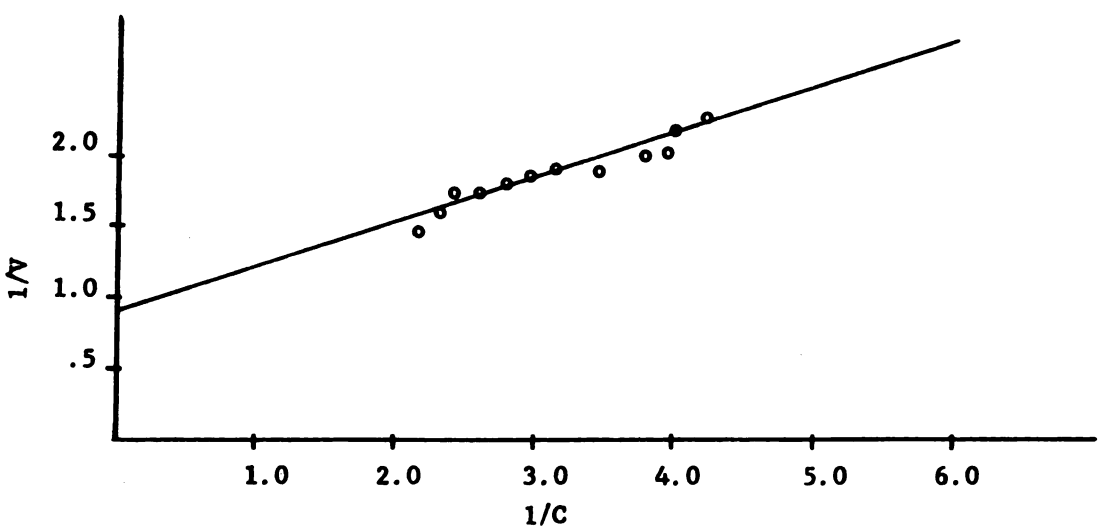

Figure 12. A plot of 1/V versus 1/C for ANS titration of CHT.

Analysis of such titration data by a Scatchard analysis requires a plot of V/C versus V and is shown in Figure 13. The intercept on the V axis is N and equals approximately 1.2. The intercept on the V/C axis is K_aN and gives a K_a of roughly $2.5 \times 10^4 M^{-1}$. These analysis clearly suggest that there is only one ANS bound per chymotrypsin molecule and that it binds with an affinity near $10^4 M^{-1}$.

E. The Activation of Chymotrypsin

Chymotrypsin is produced from its zymogen by the proteolytic action of trypsin and chymotrypsin. The main step in this enzyme activation is the tryptic cleavage of the Arg 15-Ile 16 peptide bond which produces enzymatically active π-CHT. This activation is discussed in detail in section IV. B.

From Figure 10 it is clear that at pH 7.0 the fluorescence of CG-ANS is approximately five times greater than the fluorescence of CHT-ANS at this pH. Also while the emission wavelength maxima of CG-ANS is near 475 nm, regardless of pH, CHT-ANS has an emission maxima near 505 nm at pH 7.0. Thus we should be able to follow the conversion of CG to CHT by monitoring the decrease in ANS fluorescence intensity and red shifts in its fluorescence emission maxima.

The fluorescence of CG-ANS solutions was, in fact, found to reduce to the fluorescence of CHT-ANS solutions as CG was converted to CHT by the addition of small amounts of trypsin. Figure 14 shows the change in fluorescence with one such activation, initiated by $3.3 \times 10^{-7} M$ trypsin at time t=0 and followed until no further changes were noticed at 77 minutes. Not only does the fluorescence decrease with the conversion,

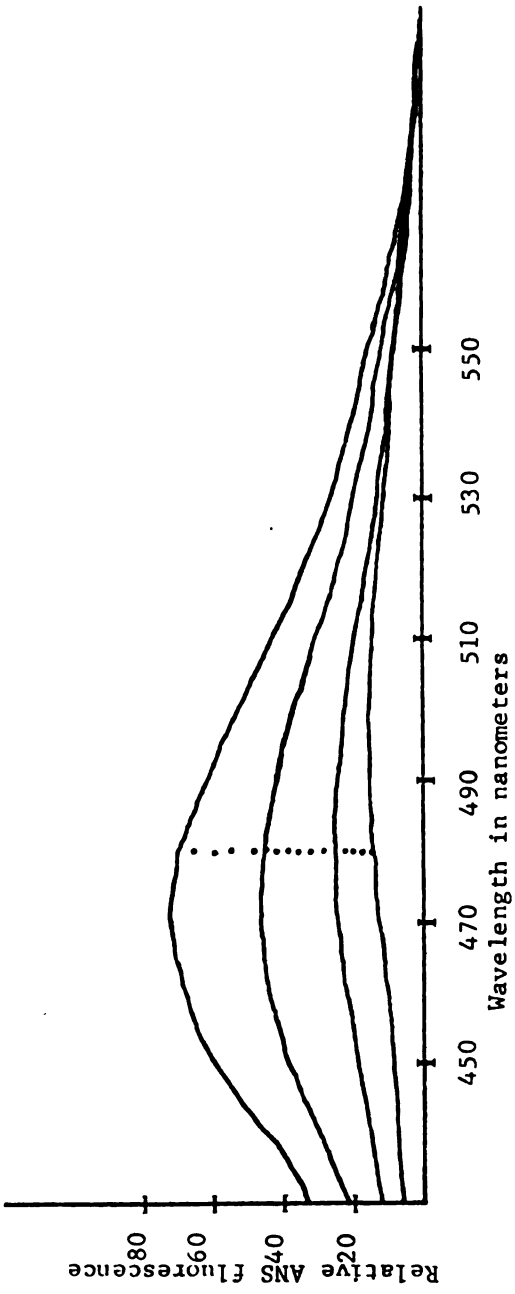

Figure 14. The change in ANS fluorescence with the conversion of CG to CHT, at time t=0, 9, 27, and 77 minutes.

the emission maxima red shifts from near 475 nm to 505 nm with time. The final emission, in terms of relative intensity and emission maxima, is that of CHT-ANS at pH 7.0.

The rate of this decrease in ANS fluorescence , in activation mixtures, was found to parallel the formation of enzymatically active chymotrypsin. Chervenka (70) has shown that solutions of CG have a greater absorption at 287 nm than solutions of CHT. He has used the decrease in absorption at this wavelength in solutions of CG, activated by trypsin, to follow the formation of π-CHT. No further changes in absorption occur when π-CHT undergoes autolysis to form the other chymotrypsins. Thus the decrease in absorption at 287 nm can be used to follow directly the formation of π-CHT from CG. In Figure 15 the conversion of 10^{-4}M CG to CHT by 3.3×10^{-7}M trypsin is shown as monitored by the decrease in ANS fluorescence at 480 nm and by the decrease in protein absorption at 287 nm. The decrease in CG-ANS fluorescence is seen to parallel the conversion of CG to π-CHT under these conditions.

The percent change of fluorescence with reaction time is somewhat greater than the formation of π-CHT as monitored by absorption changes. While π-CHT is enzymatically active, it is relatively unstable and undergoes autolysis to form the different chymotrypsins. This increased rate of change in fluorescence relative to the formation of π-CHT could result from such autolysis.

To see if autolysis was responsible for the enhanced rate of fluorescence change relative to the absorption change, the conversion was run in the presence of 0.01 M β-phenyl proprionate. BPP is known to inhibit autolysis. Figure 15 shows that in

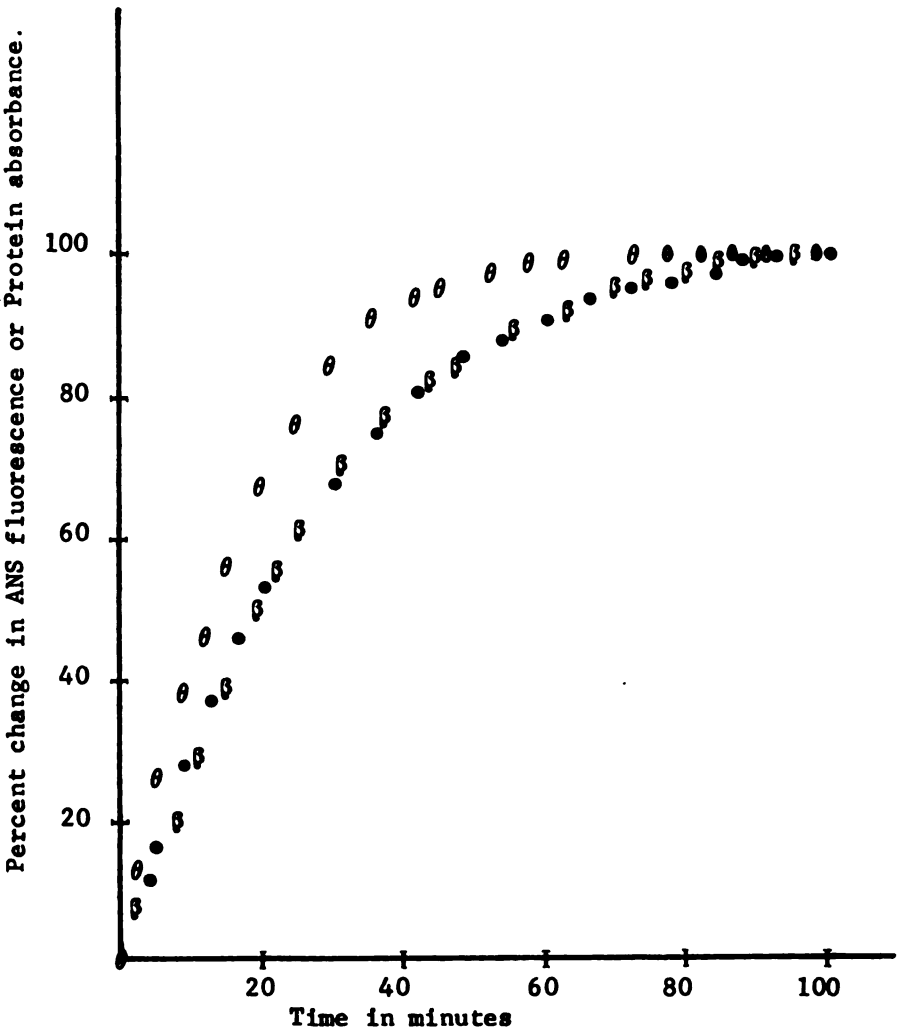

Figure 15. The decrease in ANS fluorescence (with (β) and without (θ) BPP) and the decrease in protein absorption (•), with CG activation.

the presence ofBPP the kinetics of the conversion of CG to π-CHT as monitored by the absorption change is identical with the kinetics of this conversion as monitored by the decrease in the fluorescence of the protein ANS complex. The presence of ANS itself did not affect the rate of conversion of CG to CHT as monitored by the change in protein absorption.

Commercial preparations of chymotrypsinogen contain from .2 to .5 percent residual chymotrypsin activity. Such solutions could therefore undergo a slow "autolysis" without the addition of trypsin or chymotrypsin. The addition of CHT to CG-ANS solutions was also observed to decrease the fluorescence intensity as a function of time. The rate of this decrease was much slower than when trypsin was used to activate the CG.

Thus both the tryptic cleavage of the Arg 15-Ile 16 peptide bond and to a lesser extent the chymotryptic hydrolysis of some peptide bond of CG initiate conformational changes that decrease the fluorescence of bound ANS.

F. Fluorescence Lifetimes

The interaction of polar molecules with the excited state of a fluorophore such as ANS may be thought of as a quenching reaction. Both the energy and efficiency of fluorescence is reduced relative to their values in non-polar solvents. Another parameter of fluorescence that reflects such quenching processes is the lifetime of the fluorescence excited state, τ_f. The quantum yield of fluorescence is directly related to the lifetime of the excited state: $\tau_f = \tau_o Q_f$. Where these values are as defined in section I.C. As quenching occurs and Q_f decreases, the lifetime τ_f, decreases accordingly.

Unlike fluorescence yield measurements, lifetime measurements are not hindered by scattered light, solution inner filter effects, nor do they generally have a concentration dependence. The determination of lifetimes of the fluorescence excited state of a molecule provides us with a convenient means of monitoring changes in the environment of fluorophores such as ANS.

Since rather dramatic decreases in the fluorescence intensity were observed in CHT-ANS solutions with increasing pH, we expected to see corresponding changes in the fluorescence lifetimes of CHT-ANS solutions with such pH changes. The deconvoluted fluorescence decay curves of CHT-ANS at pH 3.6 and 7.0 are shown in Figure 16. Since the slopes of these curves are proportional to the fluorescence lifetimes, it is obvious that rather large differences in lifetime exist.

Artifacts of the deconvolution analysis and perhaps due to scattering have been reported at early times after excitation in others' lifetime data analysis (28). Such artifacts appear as an early component of the decay curve and are generally on the order of a few nanoseconds. They are not believed to actually correspond to the lifetime of any fluorescence species. We therefore chose to analyze our lifetime data from channel 20 to 128 (with .383 nanoseconds per channel) after the occurance of this early component.

Analyzing in this manner we found the following: The pH 3.6 fluorescence decayed as a single exponential, with a lifetime of approximately 12.0 nanoseconds (correlation coefficient of 1.000). The pH 7.0 fluorescence decay was

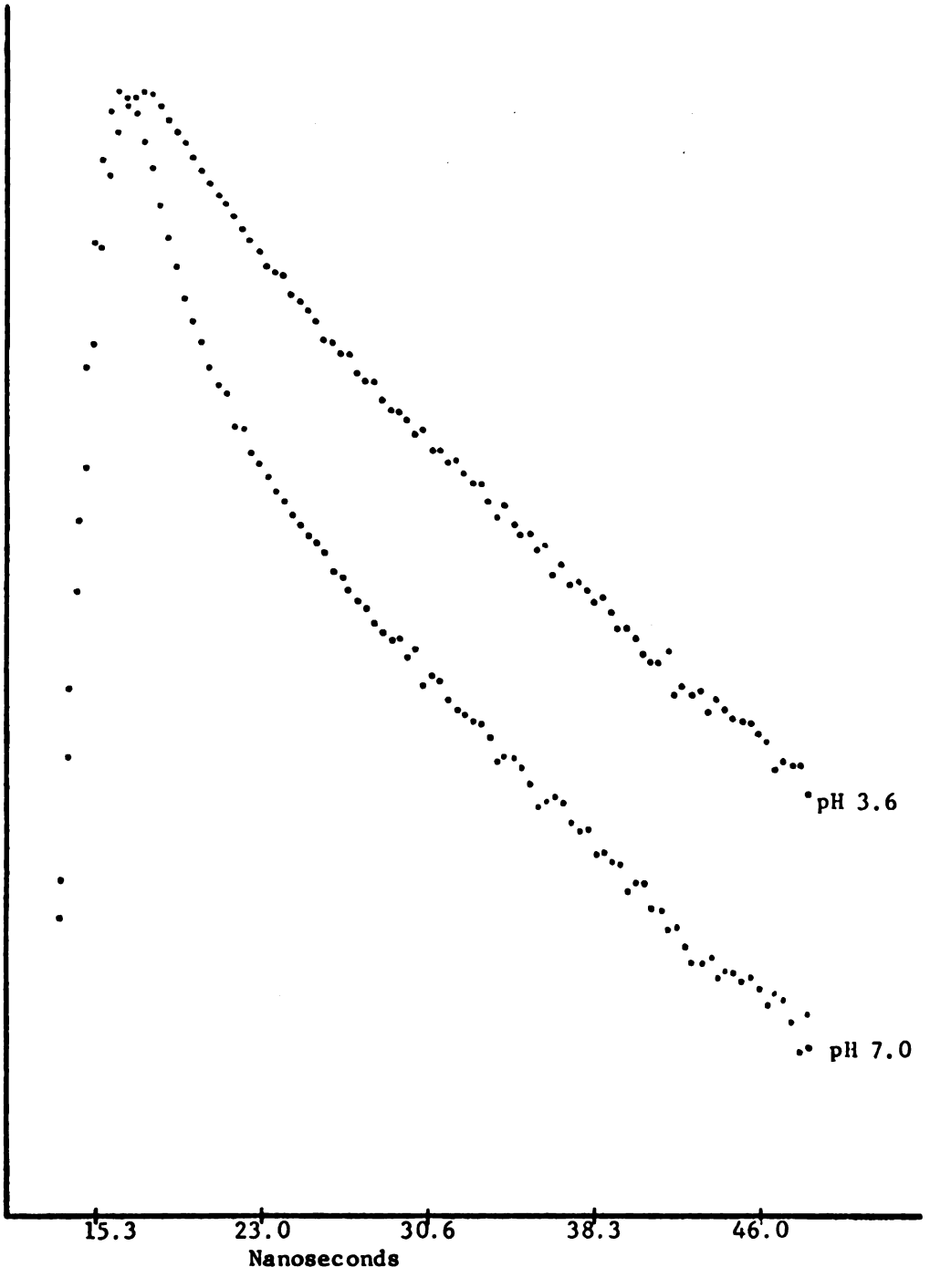

Figure 16 Fluorescence decay curves of CHT-ANS at pH 3.6 (upper) and pH 7.0 (lower).

best fitted by a double exponential decay. Channels 20-35 were best fit by a single exponential decay with a lifetime of 8.8 nanoseconds (correlation coefficient of .9999). Channels 45-128 were best fit by a single exponential decay with a lifetime of approximately 12.0 nanoseconds (correlation coefficient of 1.000).

At pH 7.0 the long lifetime component (12 ns) of the pH 3.6 decay still existed, however, a component with a reduced lifetime now contributed to the fluorescence decay curve. This additional short lifetime component may correspond to a quenched ANS species. Its presence could account for the red reduced fluorescence yields of CHT-ANS solutions at high pH.

Regardless of the method of data analysis, it is obvious from Figure 16, that as the pH is increased from 3.6 to 7.0, a short lived component shows a much greater contribution to the fluorescence decay curve. This decrease in lifetime is observed to correspond to the decrease in relative fluorescence we observed with increasing pH.

Similar changes in the fluorescence decay curves were observed with such pH changes in tosylated-CHT-ANS and CG-ANS solutions. The reduction of the lifetime is not as dramatic as the pH is raised from 3.6 to 7.0 in CG-ANS solutions as it is in CHT-ANS solutions. This is consistent with our finding that CG-ANS does not show as large of decrease in fluorescence yield as a function of increasing pH as does CHT-ANS.

Because fluorescence lifetimes are so sensitive to the environment of the fluorophore, we also used them to reexamine

the affect of several competitive inhibitors on CHT-ANS fluorescence. From our relative fluorescence yield data we saw that the presence of the competitive inhibitors BPP or tosylation of CHT did not affect CHT-ANS fluorescence. Our fluorescence lifetime data support this conclusion. CHT-ANS lifetime is not affected (still 12.0 ns at pH 3.6) by the presence of 5 x 10^{-3}M BPP or by tosylation. Addition of another competitive inhibitor of CHT, N-formyl tryptophan, does not alter the lifetime of CHT bound ANS. Thus these lifetime measurements support and confirm the conclusions we reached from our studies of relative fluorescence yields.

G. Fluorescence Depolarization

Using the nanosecond decay curves of the parallel and perpendicular components of the CHT-ANS emission and assuming CHT is roughly spherical in shape (an assumption consistent with X-ray analysis), we calculated the anisotropy of the emission of ANS bound to CHT at three different pH values. The plots of log anisotropy versus time at pH 3.6, 4.75, and 7.0 are shown in Figure 17. For a rotating sphere the slope of these plots is equal to $-3/\rho$ where ρ is the rotational relaxation time of the bound dye. If the dye is bound rigidly to the protein such that it rotates with the protein (as a protein-dye complex), then the rotational relaxation time it reports will be that for the rotation of the protein. If the dye is able to undergo some rotation of its own during the lifetime of its excited state(rotation which is free from the rotation of the protein), then the rotational relaxation time it reports would be less than that of the protein. By

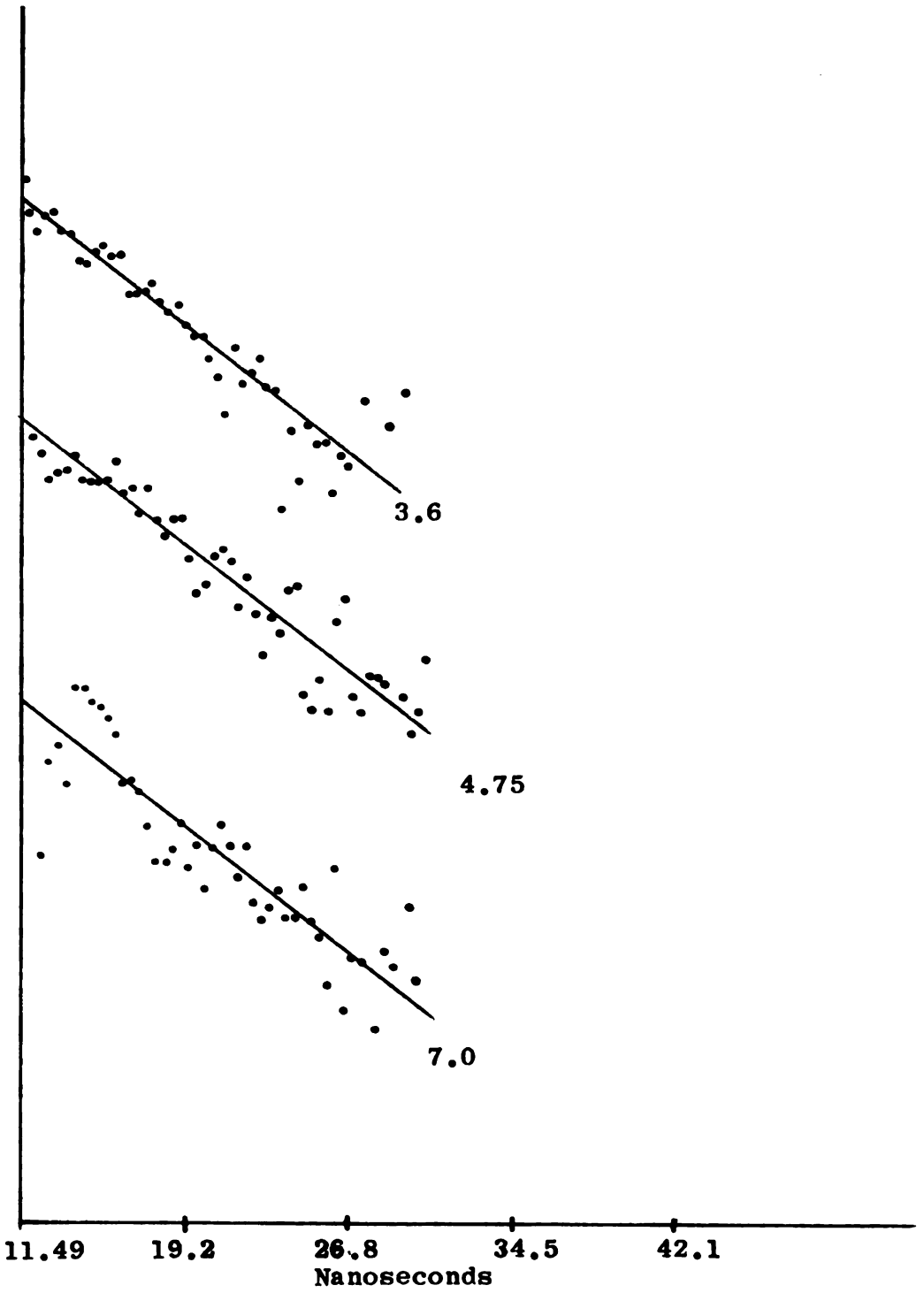

Figure 17. Log Anistropy as a function of Time for CHT-ANS at pH 3.6, 4.75, and 7.0.

comparing the rotational relaxation times of the protein with that reported by ANS bound to the protein, we get an indication of the rigidity of dye binding.

Haugland and Stryer (112) have bound a highly fluorescent anthraniloyl group covalently to CHT. They reproted that the dye rotated with the protein and calculated a rotational relaxation time of 49 nanoseconds for CHT. These results show that the rotational relaxation time for CHT itself is nearly 49 ns.

The lines drawn through the data of Figure 17 represent a rotational relaxation time of 45 nanoseconds and provide a good fit for the data at each pH. This value is within experimental error of the 49 ns rotational relaxation time for CHT. These experiments suggest that ANS is bound rigidly to CHT such that it rotates only as a protein-dye complex and that the rigidity of ANS binding to CHT is not influenced by pH in this range.

VI.

A. X-Ray Crystallographic Studies

After a full characterization of the binding of ANS to CHT in solution and with such controversies concerning the mechanism of ANS binding and fluorescence enhancements in proteins, we sought to determine the X-ray crystallographic structure of ANS and its binding site on CHT at a pH where it is highly fluorescent. We anticipated that ANS would bind to CHT crystals at the same site that it binds to CHT in solution.

The density of CHT crystals is 1.25 grams/cm^3. This corresponds to a solution containing approximately 37% mother

liquor and 0.10 M CHT within the crystal. Such crystals are refered to as "wet" crystals because of the large amount of mother liquor within them. The diffusion of heavy metal ions, substrates, inhibitors and hydrogen ions is facilitated by the presence of this mother liquor throughout the crystal. The crystalline enzyme does, in fact, exhibit some catalysis. Inhibitors of similar and larger size than ANS have been shown to diffuse into the crystal and bind at the same site on CHT as they do in solutions of CHT (i.e. N-formyl-tryptophan (49) and proflavin (113)). Thus we had reason to believe that ANS would bind to CHT in the same manner in the crystal as in solution.

ANS was allowed to diffuse into CHT crystals in their 75% saturated $(NH_3)_2SO_4$, pH 3.6 soaking solution. With the diffusion of ANS into the crystals they became highly fluorescent. Figure 18 shows an emission spectrum of CHT-ANS in solution at pH 3.6 compared to an emission spectrum of CHT crystals into which ANS had been allowed to diffuse. Both show an emission maxima near 488-490 nm. The similarity of these emission maxima suggest that ANS is in a similar environment in the crystal as in solution.

Since the quantum yield of ANS fluorescence cannot be determined from such crystals, the fluorescence lifetimes were obtained using the single photon counting technique as described previously (114,115). The fluorescence decay curves of ANS in CHT solutions and in the crystal at pH 3.6 are shown in Figure 19. Analysis of both of these decay curves yields lifetimes of 11.9 nanoseconds with a correlation coefficient of

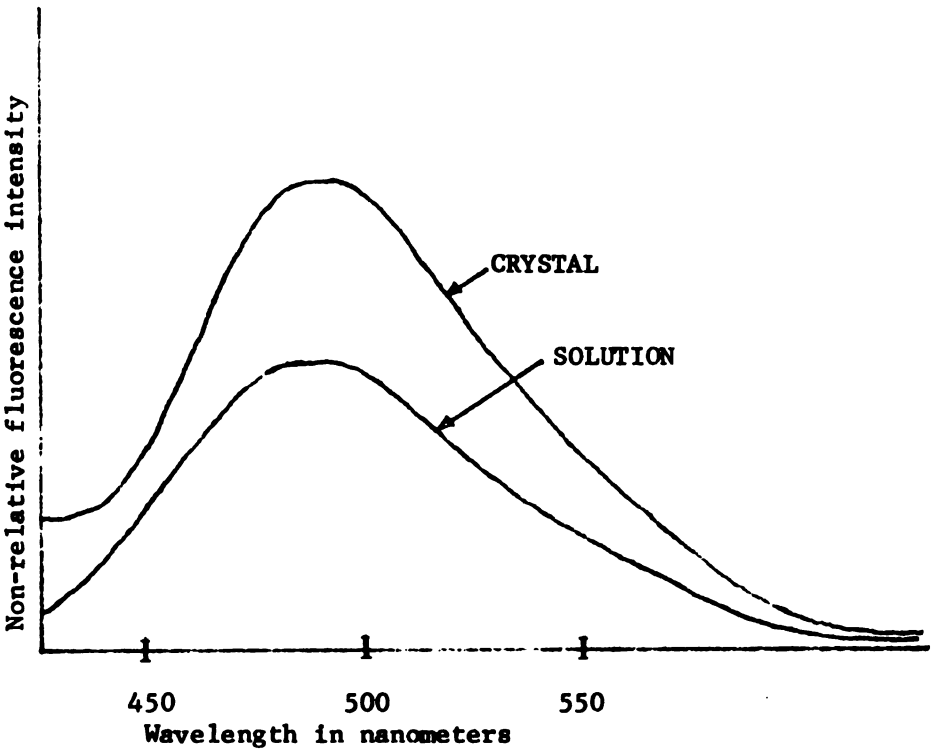

Figure 18. Emission spectra of CHT-ANS in solution and in the crystal.

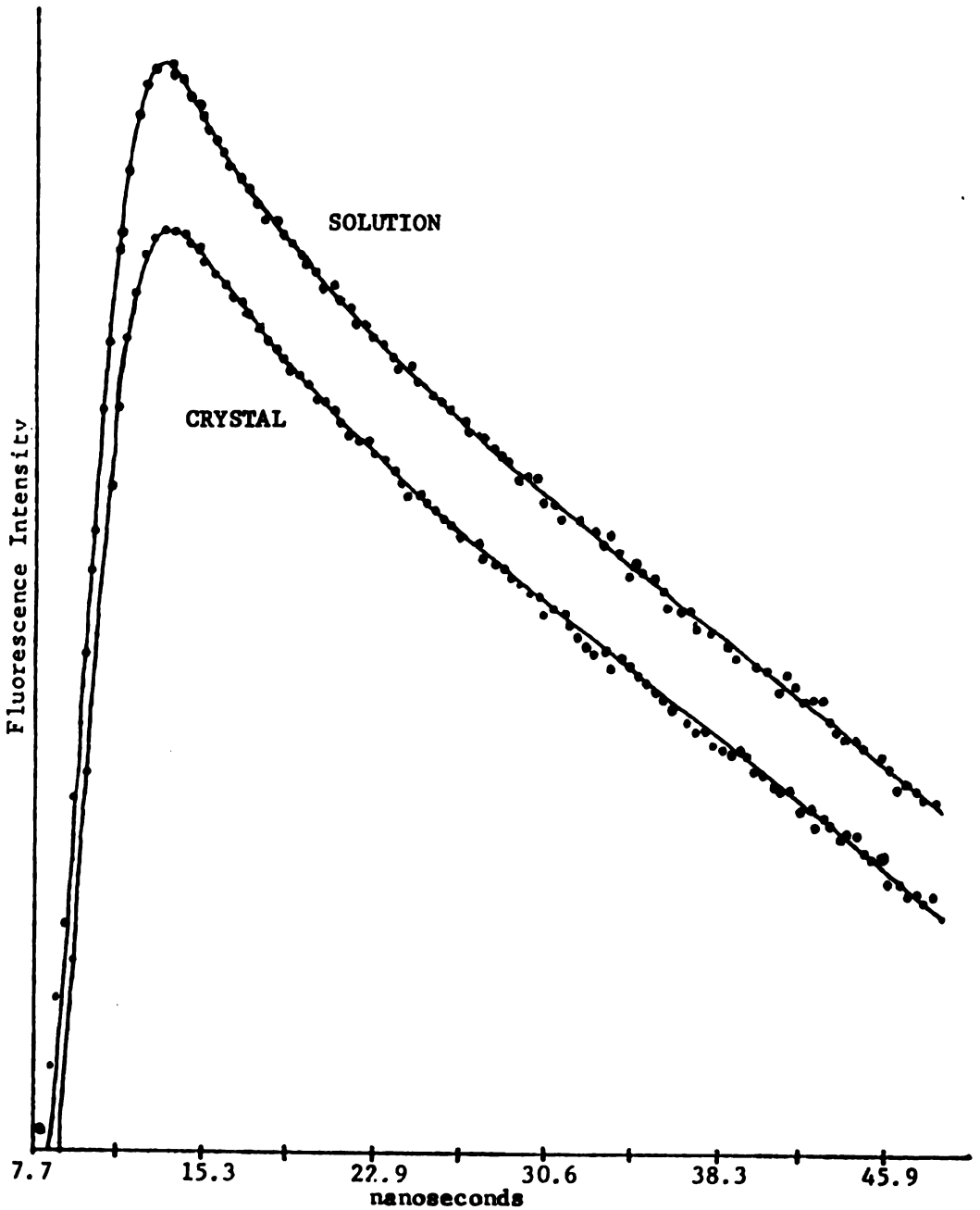

Figure 19. Fluorescence decay curves of CHT-ANS in solution and in the crystal.

.9998. Since the fluorescence lifetime is extremely sensitive to environmental conditions, these results strongly suggest that ANS is in virtually the same environment in the crystal as in solutions at pH 3.6. Thus we had very strong initial evidence that ANS bound to the same site on CHT in the crystal as in solution.

The X-ray crystallographic structure of CHT-ANS at pH 3.6 was determined by the same procedure as the CHT-inhibitor structures have been previously determined and described by Tulinsky and co-workers (42,43,105). A schematic packing diagram of CHT in the crystal, viewed along the a* direction is shown in Figure 20, taken from (96). Molecules I and I' form an asymmetric unit and are related by non-crystallographic twofold rotation axes A and B. Active site regions are denoted by * .

The first crystallographic result we obtained was a 6.0 Å centric (h0l) difference electron density projection of CHT-ANS minus native CHT. This is represented in Figure 21. The local twofold axes are shown approximately perpendicular to the C axis. Negative contours are shown broken, positive contours are solid. The large positive electron density in this two dimensional difference map arises from the bound ANS molecule.

Examination of this difference electron density projection revealed some interesting facts. The ANS binding site lies near the local twofold axis, Dyad B. It is quite distinct and seperated from the active site region of CHT (which is located about Dyad A). Since the unit cell contains four CHT molecules it is not clear from projection that only one ANS molecule binds

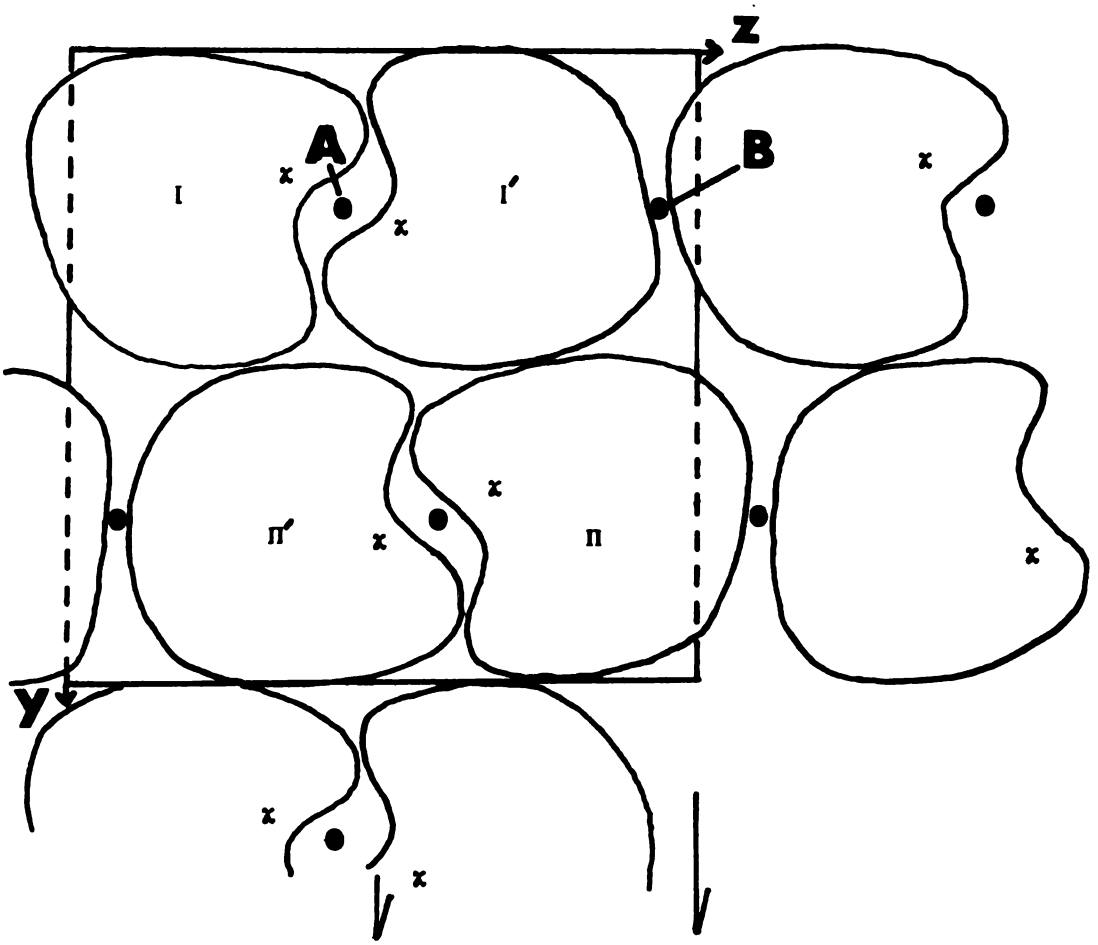

Figure 20. Schematic packing diagram of CHT in the crystalline state.

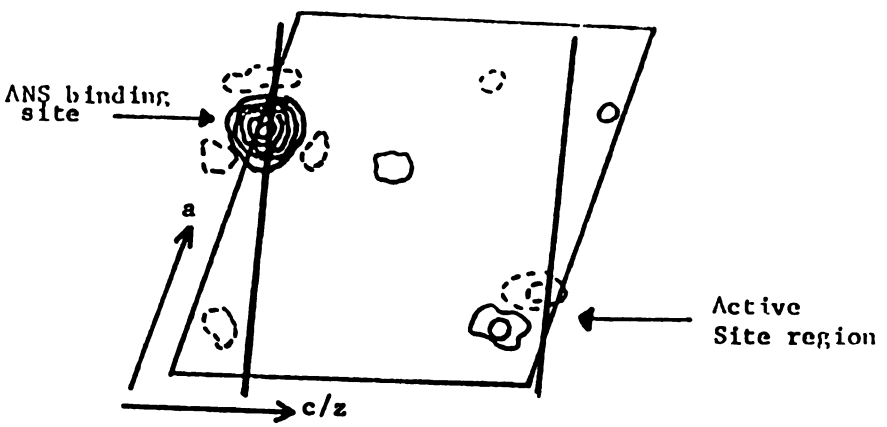

Figure 21. Centric [h0l] difference electron density projection of CHT-ANS minus native CHT.

per CHT in these crystals. Aside from the ANS binding site itself, only very minor perturbations of native structure are produced by ANS binding. This may be seen by the lack of any large electron density changes away from the ANS binding region in Figure 22. These results were all in agreement with what we had learned about ANS binding to CHT in solution.

Since we had a good degree of substitution of ANS in its binding site on crystalline CHT, a three dimensional data collection and analysis at 2.8 Å resolution was conducted on the same crystal. This three dimensional analysis revealed the exact position and geometry of ANS and its binding site on CHT at a very high resolution.

The electron density of ANS (as determined from the CHT-ANS minus native CHT difference electron density map) is shown in relation to the electron density of native CHT in Figure 22. The (+) represents the local twofold axis,dyad B, as it is projected along the a* axis. The ANS molecule shows good twofold symmetry about dyad B, thus establishing that one ANS molecule binds to each monomer of the dimer of CHT. Again this supports our solution studies which indicate one ANS per CHT monomer.

There are only two contacts of native protein electron density with ANS electron density. The most extensive of these is the overlap of the cysteine 1-122 disulfide bridge electron density with ANS. The other contact of protein-ANS electron density comes from glutamine 239 (Gln 239') of the twofold related neighboring molecule (an intermolecular interaction present only in the crystal). Gln 239' is a part of the helix

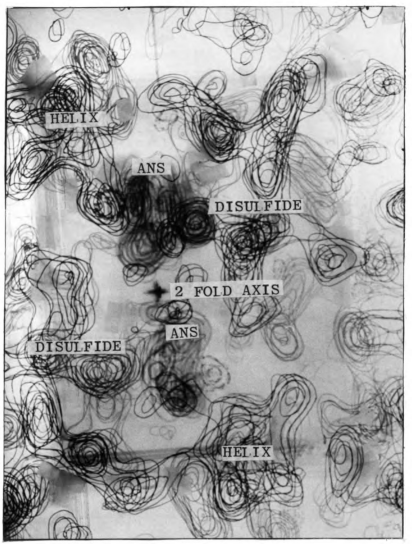

Figure 22. Electron density profile of CHT-ANS.

formed by residues 235-245 of the carboxyl terminal of the C chain. The side chain of Gln 239' lies off of this helix and comes in close contact with the naphthalene ring of ANS.

Comparison of these electron densities gives us an idea of the closeness of these contacts of native CHT with the ANS molecule. The sulfur atom of the sulfonate and nitrogen atom of ANS lie just less than 3.0 Å from a sulfur atom of cysteine 1. The center of the naphthalene ring (ring 1) of ANS lies less than 3.0 Å away from the sulfur atom of cysteine 122.

Most regions of the carboxyl terminal helical region are quite distant from the ANS molecule (relative to its immediate proximity to the Cys1-Cys122 disulfide). Only the side chain of Gln 239' shows any appreciable overlap of electron densities with ANS. This sidechain lies near the back (carbon atoms 4 and 5) of the naphthalene rings of ANS. The nitrogen atom of the NH_2 group of the Gln 239' sidechain is approximately 3.0 Å from C_4 of the naphthalene ring of ANS. The oxygen of the carboxyl group of this sidechain is approximately 3.7 Å from C_4 of the ANS. Both the oxygen of this carboxyl and nitrogen of the NH_2 of this sidechain are far removed from the chemically reactive NH and SO_3^- groups of ANS (7.6 and 6.2 Å away from the sulfonate of ANS, respectively)

While the changes in protein structure with ANS binding will be discussed in detail later, it should be mentioned that the CHT-ANS minus native CHT difference map shows negative and positive electron density changes associated with Gln 239'. The changes show that upon ANS binding, Gln 239' apparently moves away, along dyad B and in the x direction, from the naphthalene ring system

of ANS. Such changes occur over approximately 4.0 Å. After Gln 239' is removed from the ANS region, no close interactions of ANS and helical residues of CHT occur. The only remaining close interaction of probe and protein is that in the region of the Cys 1-122 disulfide bridge.

The closest interactions of ANS with the disulfide binding region and the helical regions of native CHT determined from the 2.8 Å resolution CHT-ANS minus native CHT difference map are listed in Table 2. In these distances, as listed, considerations of the changes in CHT structure with ANS binding were not made. Therefore they represent only the position of ANS relative to native CHT.

The binding of ANS to CHT in the crystal is represented in Figure 23. P represents the ANS molecule. The dimer between molecules 1 and 2 arises from dyad A. Two dimers are related (molecules 2 and 3) by dyad B. ANS has one binding site on each CHT monomer and its binding to this site shows a twofold symmetry, as do CHT molecules. ANS binds to the "back" of the CHT molecule with respect to its active site.

Because the ANS binding site lies in an intermolecular region of the CHT crystal we were faced with a problem concerning the actual ANS binding site on CHT. The CHT molecules in the crystal are rather close, spacially, about dyad B and it was difficult to tell to which protein molecule and therefore to which site the ANS was actually bound. For example, was the top ANS molecule in Figure 23, bound in a very close tight complex to CHT 3 or in a looser complex with protruding residues of the CHT 2 molecule ? The ANS binding site for the top ANS molecule on CHT 3 corresponds

Table 2. Distances between ANS and specific amino acids of CHT.

ANS Atom	Amino Acid	Atom	Distance (Å)
N	CYS 1	Sulfur	2.9
S	CYS 1	Sulfur	3.0
S	CYS 1	Nof NH_3	5.0
N	CYS 122	Sulfur	3.5
S	CYS 122	Sulfur	3.3
N	VAL 3	C of CH_3	5.0
N	LYS 203	N of NH_3	9.6
S	LYS 203	N of NH_3	9.0
N	ASN 204	O of C=O	6.5
S	ASN 204	O of C=O	6.8
N	ASN 204	N of NH_2	8.5
S	ASN 204	N of NH_2	8.5
N	THR 208	βCarbon	9.0
S	THR 208	βCarbon	9.0
C_5	THR 208	βCarbon	5.5
N	ALA 206	C of CH_3	5.4
N	ASP 48'	βCarbon	6.2
S	ASP 48'	βCarbon	5.6
S	ASP 48'	O of C=O	7.3
S	ASP 48'	N of NH_2	5.5
N	LEU 123'	βCarbon	7.0
S	LEU 123'	βCarbon	5.5
N	VAL 235'	βCarbon	9.4
S	VAL 235'	βCarbon	9.4
C_4	VAL 235'	βCarbon	7.0
N	VAL 238'	βCarbon	8.6
S	VAL 238'	βCarbon	8.5
N	VAL 238'	C of CH_3	7.5
N	GLN 239'	N of NH	6.2
N	GLN 239'	O of C=O	7.2
S	GLN 239'	N of NH	6.2
S	GLN 239'	O of C=O	7.6
C_4	GLN 239'	N of NH	3.0
C_4	GLN 239'	O of C=O	3.7
N	LEU 242'	βCarbon	5.0
S	LEU 242'	βCarbon	7.0
N	LEU 242'	C of CH_3	3.6

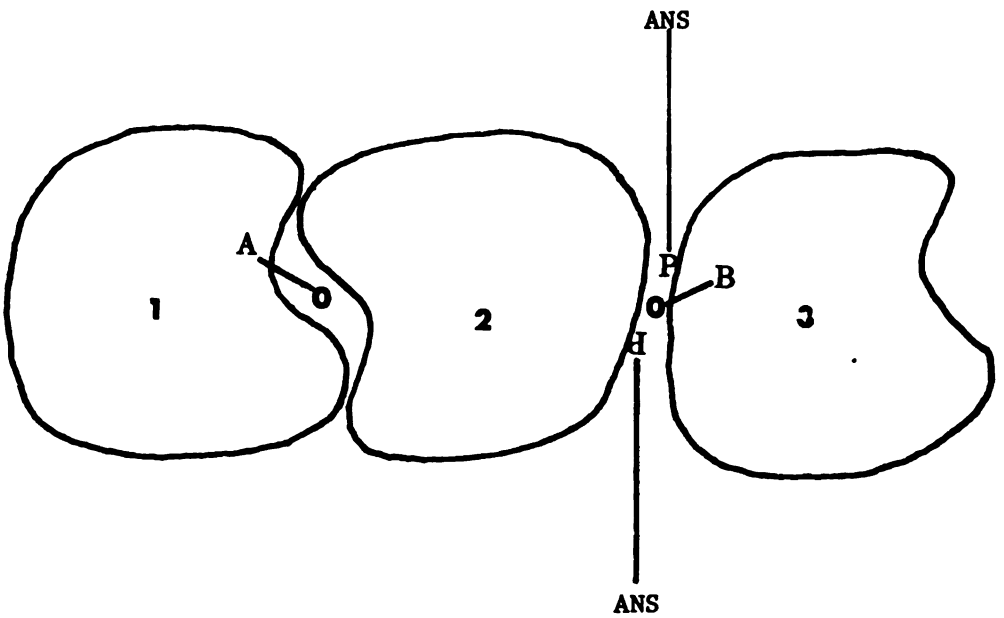

Figure 23. Schematic diagram of ANS binding site in the CHT crystal.

to the Cys 1-122 disulfide bridge region. The ANS binding site for the top ANS molecule on CHT 2, however, corresponds to the area below the disulfide bridge and above the helical region of the carboxyl terminal C chain. From proximity alone, ANS is closer to the disulfide bridge than to the helical region. Only the sidechain of Gln 239' of the helix of CHT 2 is close to the ANS molecule. This closeness is removed upon ANS binding and only the very close interaction of ANS with the disulfide region remains.

We might expect on the basis of such a proximity argument that the ANS binding site was near the Cys 1-122 disulfide bridge. Then the more distant relation of ANS to the helical region of the neighboring CHT molecule (2) would be only a consequence of the closeness of CHT molecules about dyad B. The removal of Gln 239' from the region of ANS electron density upon ANS binding also supports this hypothesis. However, we felt that additional evidence was needed to establish conclusively at which of these two sites ANS was actually bound.

When the electron densities of ANS on native CHT are fit to a molecular model of native CHT the relation of ANS to these particular residues on native CHT becomes apparent. The relationship between possible ANS binding sites on CHT is shown in Figure 24. Dyad B is shown between ANS molecules and the view is looking from the surface of the molecule into the protein along the Z direction.

The top ANS represents the relationship of ANS as it binds to CHT 3, of Figure 23, that is if the Cys 1-122 disulfide region is the actual binding site. The bottom ANS represents the ANS

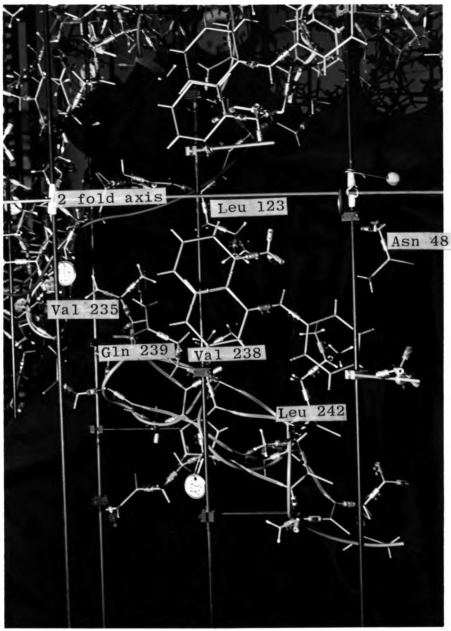

Figure 24. A Molecular Model of ANS bound to CHT.

binding site if it binds over a larger distance to CHT 2, that is, if the helical region is the actual binding site. A closer view of the ANS interaction with the disulfide region of CHT 3 is shown in Figure 25.

By considering these two-dimensional pictures and distances of the specific amino acid residues from the ANS molecule, as listed in Table 2, one can get a three-dimensional feeling of the relationship of ANS to each of these regions of CHT. Again, the interactions of the ANS molecule is closer to the disulfide region and CHT 3 than to the helical region and CHT 2. However, we sought more than such proximity evidence alone to establish to which CHT molecule and to which site the ANS was bound.

B. Fluorescence Perturbation Studies

In order to definitely decide which site was the binding site we needed to selectively perturb one site over the other and look for the corresponding change (or lack of change) in ANS fluorescence. A heavy metal used in the heavy atom isomorphous replacement method for phase determinations in CHT crystal structure determination fulfilled this need.

X-ray structural work had shown that the heavy metal anions $PtI_4^=$ and $PtCl_4^=$ (refered to as PtI4 and PtCl4 in the remainder of the text) bind to specific residues on CHT (42,116,117,118). These haloplatinates were found to bind to three specific sites in CHT. At one site the haloplatinate interacts specifically with the terminal amino group of Cys 1 and with at least one of the two sulfur atoms of the Cys 1-122 disulfide. The other two platinum binding sites are located about dyad A and are near the active site residues. One site is near the sulfur of

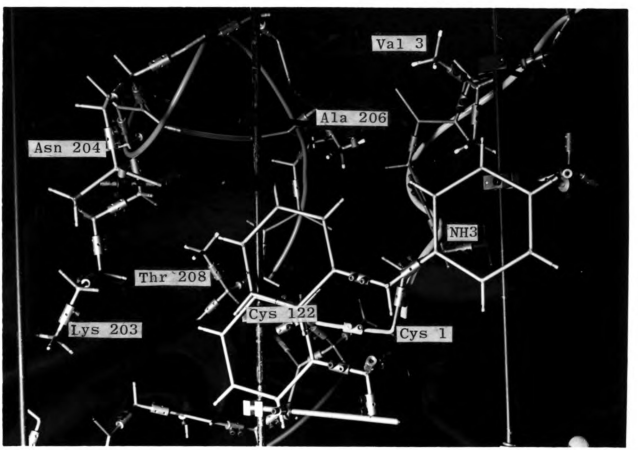

Figure 25. A Molecular Model of the ANS binding site on CHT.

atoms of the methionine 192 side chains of two adjacent molecules. The binding of platinum to the third site (near the active site) is not clearly understood (117). In each case the binding of the platinum anions to CHT seems to involve the interaction of a heavy metal ion with a sulfur containing group. This is consistent with the co-ordination behavior of platinum and sulpho-organic compounds (119, 120).

Blow et al. (118) have shown that the PtI4 that gave the highest occupancy is the site near the Cys 1-122 disulfide. Consistent with this Tulinsky et al. (42) have isolated two PtI4 derivatives as a function of anion concentration. One derivative corresponds to a 15:1 molar excess ratio of PtI4 to enzyme and the other, Pt(+), derivative to a 25:1 molar excess ratio of platinum. Intense PtI4 substitution is observed at the Cys 1-122 binding site in the platinum minus native difference map. As the concentration of PtI4 is increased the Pt(+) minus platinum difference map shows even greater specific substitution at the site near the Cys 1-122 disulfide.

The specific binding of PtI4 to the region near the Cys 1-122 disulfide and to two other distant sites was the type of specific perturbation that was needed to determine if ANS was bound near the Cys 1-122 disulfide or near the helical region of the carboxyl terminal C chain. If ANS were bound near the disulfide in solution, then PtI4 should perturb its fluorescence. If the ANS were bound approximately 10Å below this site, in the helical region, then the binding of PtI4 should not affect its fluorescence.

ANS fluorescence in solutions of CHT is observed to be drastically perturbed by very low concentrations of PtI4.

Figure 26 shows the fluorescence decay curves of 10^{-4}M CHT + $5x10^{-5}$M ANS native, at pH 3.6, and in the presence of $1.28x10^{-5}$M $3.84x10^{-5}$M and $1.28x10^{-4}$M PtI4. The fluorescence lifetime is observed to be reduced by .40, .70, 3.2, and 4.5 nanoseconds by the addition of $1.28x10^{-5}$M, $3.85x10^{-5}$M, $6.4x10^{-5}$M and $1.28x10^{-4}$M PtI4, respectively. Thus the fluorescence lifetime of CHT-ANS is reduced more than one third of its original value by additions of very small amounts of PtI4.

The fluorescence intensity is of course also decreased with the addition of PtI4. However, since the absorption of PtI4 significantly overlaps ANS absorption and the absorption of PtI4 changes with binding, interpretation of fluorescence intensity changes are hindered. Lifetime determinations which are not affected by such absorption affects are much more reliable indicies of changes in ANS environment than fluorescence intensity data.

In the crystalline isomorphous replacement method, PtI4 binding occured under conditions where the crystals were stabilized: pH 3.6 and high concentrations of $(NH_4)_2SO_4$. For further assurance that the PtI4 was binding as in the crystal, the PtI4 experiment was repeated in the presence of 25% and 50% $(NH_4)_2SO_4$. In this case the lifetime was decreased by nearly 6.0 nanoseconds by the addition of $1.28x10^{-4}$M PtI4. Similar quenching of tosylated-CHT-ANS fluorescence by PtI4 was also observed.

These experiments show conclusively that a dramatic quenching of ANS fluorescence in CHT-ANS solutions occurs with the addition of small amounts of a molecule which binds to the sulfur atoms of the Cys 1-122 disulfide bridge. This quenching of ANS fluorescence could presumably occur by two mechanisms.

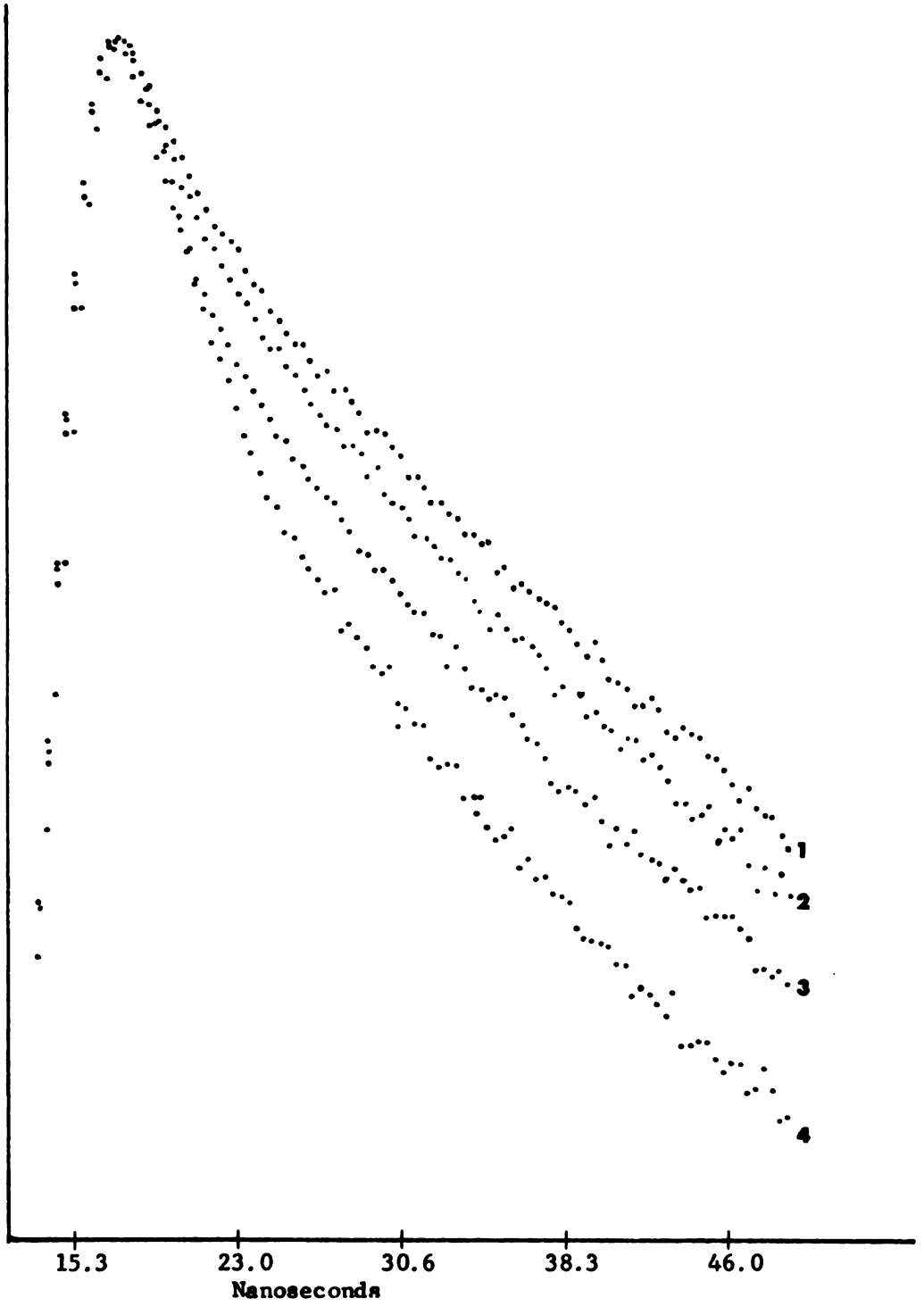

Figure 26. Fluorescence decay curves of CHT-ANS native and in the presence of increasing concentrations of K_2PtI_4 .

First, the binding of platinum near the ANS binding site on CHT could quench ANS fluorescence by competitively inhibiting ANS binding. If ANS were removed from its binding site on CHT to an aqueous solution, its fluorescent yield and lifetime would decrease dramatically. Second, heavy metal atoms are known to collisionally quench the fluorescence of fluorophores in solution. This phenomena is known as the heavy atom effect.

Quenching by the heavy atom effect requires a molecular collision of the heavy metals with the excited state fluorophore (121). Such collisions increase the non-radiative process of intersystem crossing and depopulate the excited state of the fluorophore. Because actual molecular collisions with the excited state molecules are required for such quenching, high concentrations of quencher (heavy atom) and fluorophore are required. When the fluorophore and heavy atom are both free in solution, concentrations of each on the order of 10^{-3} of 10^{-2}M and above are required for quenching by this mechanism.

Since we were observing large quenching effects with $5x10^{-5}$M ANS and PtI4 in CHT solutions, we did not feel that random collisions of PtI4 in solution with ANS bound to CHT could be responsible for such effects. However, as a control we decided to test the concentration dependence of the heavy atom effect of PtI4 on the fluorescence of free ANS in solutions. To do this successive increments of PtI4 were added to a highly fluorescent solution of $5x10^{-5}$M ANS in n-propyl alcohol. The fluorescence decay curves in the absence and in the presence of $3.8x10^{-4}$M and $3.8x10^{-3}$M PtI4 are shown in Figure 27. The lifetime for the ANS in n-propyl alcohol in the absence of PtI4 is

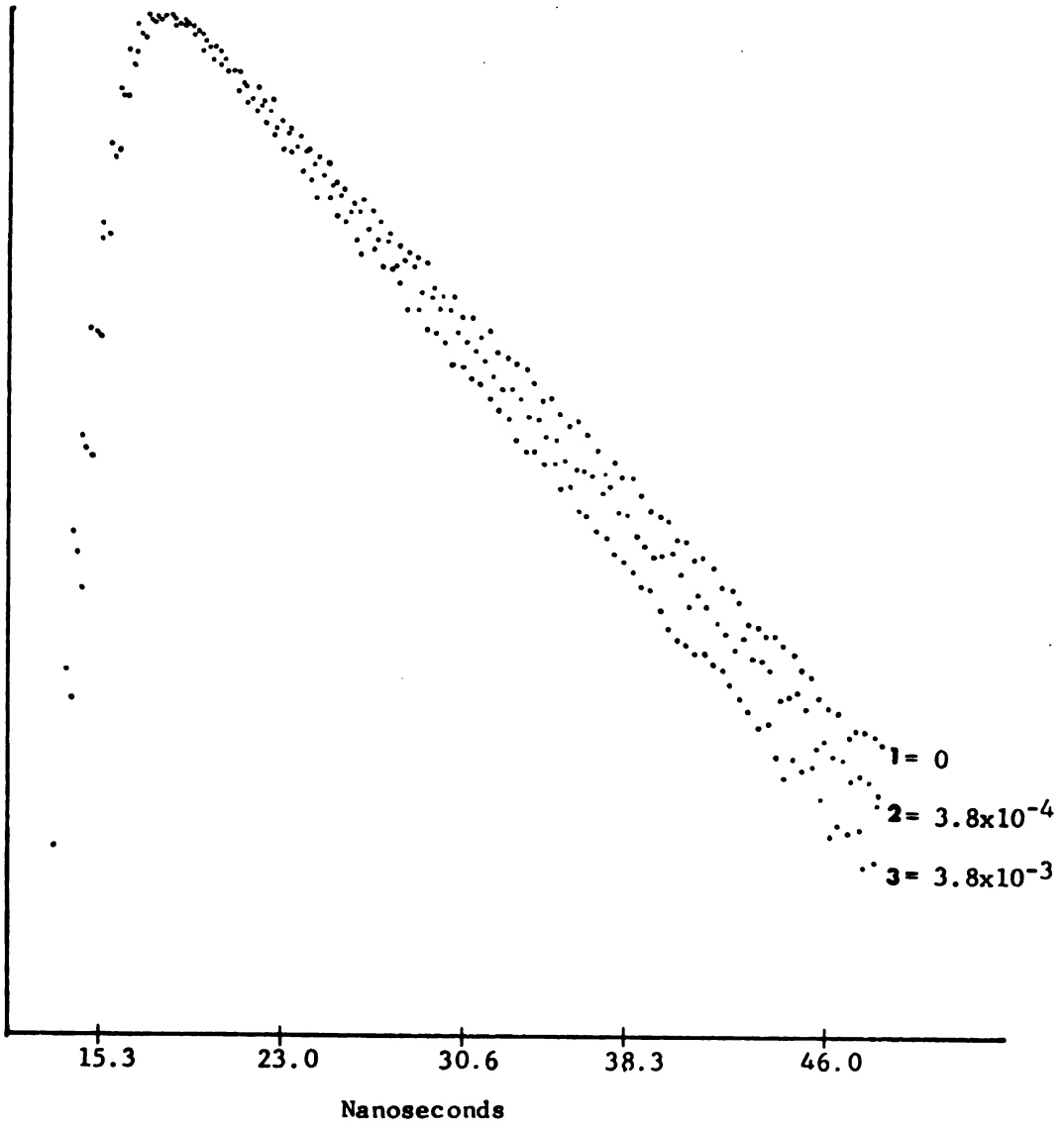

Figure 27. Fluorescence decay curves of ANS in n-propyl alcohol with increasing concentrations of K_2PtI_4 .

10.20 nanoseconds. This lifetime is reduced by 0.70 nanoseconds by the addition of 3.8×10^{-4}M PtI4 and by 1.21 nanoseconds by the addition of 3.8×10^{-3}M PtI4. From this study it is clear that very high concentrations of PtI4 are required to exhibit even small quenching effects on free ANS in solution. (i.e., 3.8×10^{-3} M PtI4 decreased the lifetime of ANS in solution by only 1.2 nanoseconds, while in CHT-ANS solutions nearly thirty times less PtI4 reduced the lifetime of ANS by approximately 5 nanoseconds.)

To further rule out the possibility of random collisional quenching by heavy atoms in CHT-ANS solutions we wanted to test what concentrations of a known heavy atom quencher were necessary to quench ANS fluorescence in CHT solutions. Iodide ions are known to be very effective heavy atom quenchers of the fluorescence of aromatic fluorophores (122,123). While the fluorescence of ANS in n-propyl alcohol was reduced by saturating conditions of potassium iodide (KI), no effect on the fluorescence of ANS in CHT solutions was observed even in the presence of 10^{-3}M KI.

Both of thse results clearly suggest that with the small concentrations of PtI4 used in the fluorescence perturbation experiments, no significant contribution to the observed reduction in the fluorescence or fluorescence lifetime could come from the random collisions of free PtI4 in solution with ANS bound to CHT. In addition to this, at the concentrations of PtI4 and CHT used, nearly all of the PtI4 should be bound to CHT and very little would remain free in solution. If the fluorescence of bound ANS is being decreased by the heavy atom effect, such a dramatic quenching must result from the binding of PtI4 to the ANS binding site of CHT such that the platinum is close enough to the bound

ANS that actual molecular collision occur, with great efficiency and thereby quench ANS fluorescence. This would require that PtI4 be bound at the ANS binding site.

For PtI4 to quench ANS fluorescence by competitive inhibition of ANS binding or by collisional quenching the PtI4 must be bound to essentially the same site as the ANS molecule and displace ANS or collisionally quench its fluorescence. Since PtI4 is known to be bound (probably covalently) to at least one of the sulfur atoms of the Cys 1-122 disulfide, then for ANS fluorescence to be so dramatically perturbed, ANS must also be bound to this site on the protein. Thus by such fluorescence perturbation we could clearly and conclusively determine that the ANS binding site on CHT was the site near the Cys 1-122 disulfide and that the apparent binding site near the helical region of the carboxyl terminal C chain was only a consequence of this site being adjacent to the actual ANS binding site in the twofold related molecule in CHT crystals.

C. The ANS Binding Site on Chymotrypsin

Thus ANS binds to CHT as shown in Figure 25, near the Cys 1-122 disulfide bridge and it fluoresces intensely while bound at this site. These studies also show that the ANS binding site is the same in the crystal as in solution. This is because PtI4 is known to bind near the ANS binding site in the crystal (by X-ray analysis) and in solution (by the observed fluorescence perturbation of CHT-ANS fluorescence by very low concentrations of PtI4). Thus ANS is bound both to CHT crystals and to CHT in solution at the same site and this is the region of the Cys 1-122 disulfide bridge.

D. Changes in Protein Structure with ANS Binding

While no large changes in protein structure occur with the binding of ANS to CHT, some small localized changes occur in the ANS binding region of CHT. These changes are observed in the CHT-ANS minus native CHT difference electron density map.

When ANS is positioned on native CHT it is seen to be bound near the Cys 1-122 disulfide bridge. The only close interaction from the adjacent molecule is that with Gln 239'. Their relation to one another may be seen in Figure 24. Gln 239' lies near the naphthalene ring of the ANS molecule with its NH_2 and C=O groups positioned away from ANS. In the CHT-ANS minus native CHT difference map negative electron densities occur at the position of Gln 239' and corresponding positive electron densities occur at lesser x values. These changes indicate a movement of Gln 239' away from the ANS molecule towards the sidechain of asparagine 204. These changes leave the ANS in its close interaction with the Cys 1-122 disulfide but no close interactions between ANS and the twofold related CHT molecule remain. Thus in the crystal ANS is bound near this disulfide and is surrounded by mother liquor on its exterior.

Other smaller changes occur upon ANS binding in this region. Small movements in the peptide chain region between Asn 204, Lys 203 and Lys 202 and in the side chains of Asn 204 and Lys 203 are observed. While it is difficult to interpret static changes in electron density in terms of which movements are inducing or resulting from other movements, some interpretations are more plausable than others.

ANS binding might be expected to "cause" the movement of

the highly polar Gln 239' side chain away from its non-polar naphthalene ring. The Gln 239' then moves into lesser x, away from the rings of ANS and towards the sidechain of Asn 204. This Asn 204 sidechain then approaches the sidechain of Lys 203 (charged by its NH_3). This movement of Asn 204 might induce the small changes observed in the Lys 203 sidechain and the move in the peptide linkage between Asn 204-Lys 203-Lys 202. These latter movements are very small and occur only over less than 2.0 Å.

Thus while no major rearrangement of protein structure occurs, these minor changes are observed in the ANS binding site region. It should be pointed out that no movement in the 1-122 disulfide or residues 1-3 of the A chain is observed with ANS binding. No other changes of any significance occur with ANS binding. The ANS molecule binds to CHT very much as pictured in Figure 25. Aside from the removal of the Gln 239' of the neighboring CHT molecule from the region of the naphthalene rings of ANS and small changes in the residues of the C chain (Asn 204, Lys 203, and Lys 202) little change from the native structure is observed with the binding of ANS.

E. The Conformation of ANS while Bound to Chymotrypsin

By considering the electron density profile of ANS from the CHT-ANS minus native CHT difference map, we could get an idea of the conformation of ANS while bound to CHT. While at 2.8 Å resolution we cannot comment precisely on the exact molecular orientations of the probe molecule, two features do stand out. First, the naphthalene and anilino rings appear to be nearly co-planar. Second, there is an unusual puckering of

electron density of the naphthalene ring. This makes the electron density of the naphthalene ring assume a nearly kidney bean shape. Ring 1, shows a bend or pucker out of the plane formed by the top ring (ring 2) of the naphthalene and the anilino ring. This pucker is away from the protein side of the ring. That is, the bottom naphthalene ring puckers away from the Cys 1-122 disulfide bridge. The overall conformation of ANS is as shown in Figure 25 which represents ANS bound to CHT.

We do not feel that the conformation we observe of CHT bound ANS is either of the two conformers of crystalline ANS reported by Cody and Hazel (21). In their conformers the naphthalene and anilino rings were out of plane with one another by 63 and 53 degrees for ANS(1) and ANS(2), respectively. It appears that the conformation of ANS while bound to CHT is such that the naphthalene and anilino rings are very nearly co-planar.

VII. DISCUSSION

These solution studies indicate that both CHT and CG have a pH dependent ANS binding site. This site is distinct from the active site of CHT and ANS binding does not affect chymotryptic activity nor the tryptic conversion of CG to CHT. Inhibition of enzyme activity by the presence of substrate analogues or by tosylation does not affect ANS binding. The large fluorescence enhancement at low pH is not altered by dimerization of CHT since identical fluorescence-pH profiles are observed for native and tosylated CHT (which does not dimerize at low pH (124)).

The similarities of the binding constants of CHT-ANS at both low and neutral pH suggest that ANS is bound with the

same apparent affinity under both conditions. The fluorescence depolarization data support this conclusion by showing that ANS is bound with the same rigidity at several different pH values. Therefore the fluorescence changes that are observed with pH do not result from changes in ANS binding but rather from environmental changes about bound ANS.

These structural changes within the ANS binding site must enhance the ability of polar molecules (of the protein or of solvent) to interact with ANS during the lifetime of its excited state. An increase in such interactions results in the decrease in energy and intensity of ANS fluorescence and the decrease in its fluorescence lifetime.

The conformation of CHT is quite sensitive to the H^+ion concentration of its surrounding medium. Many physicochemical techniques have been used to detect and characterize particular aspects of these pH induced conformation changes. More recently Tulinsky and his co-workers (96,105,106) have conducted high resolution X-ray crystallographic studies of CHT at several different pH values. These studies revealed several different pH conformers, changes in the total charge of the protein, localized changes in the region of certain ionizable residues, and large conformational changes which occur throughout the entire CHT molecule.

Conformational changes also occur in the tryptic conversion of CG to CHT. These changes too have been monitored by various physical probes of structure and detailed by comparison of the X-ray structure of CHT with CG (81,82).

The structural changes that occur with pH and with zymogen

activation are each very detailed and complex. Without knowing the precise location of the ANS binding site on these proteins we can not say with any certainty which aspects of these changes the ANS molecule reports.

The structurally similar fluorescent probe molecule TNS has been reported to bind to both CG and CHT with an affinity similar to that which we report for ANS (125). However, quite different phenomena were observed with TNS binding. TNS non-competitively inhibits CHT activity and substrate binding to CHT results in a large decrease of TNS fluorescence. Distinctly different pH profiles of TNS fluorescence were reported and the conversion of CG to CHT resulted in large increases in TNS fluorescence without a change in its wavelength emission maxima. These differences in probe behavior suggest that although structurally similar, these two molecules are bound to different sites on CHT and monitor different aspects of these conformational changes. In accordance with this 2,6 ANS and 2,6 TNS have quite different binding properties in other protein systems also (126).

Ten small hydrophobic molecules of the form 3-(n-alkanoyl)-O-benzoate have been reported to bind to CHT at a hydrophobic site other than the active site and to inhibit chymotryptic activity (127). The more hydrophobic and anionic the molecules of this series, the greater the observed inhibition. These investigators suggest that there exist a secondary site on CHT that is hydrophobic in nature and may regulate chymotryptic activity through the binding of small hydrophobic molecules. Since TNS is hydrophobic and anionic and inhibits activity, it may be bound to this control site. Again, we feel that ANS is

bound to yet another site on the protein such that it exhibits different fluorescence behavior and does not interfere with chymotryptic hydrolysis.

Our observations from solution studies, that ANS binds to a single specific site, which is distinct from the active site and sensitive to pH induced conformational changes in CHT have been verified by crystallographic studies of CHT-ANS crystals. Fluorescence perturbation studies of CHT-ANS fluorescence with PtI_4 and the identical fluorescence lifetimes of ANS in solutions and crystals of CHT verify that ANS binds to the same specific site and enjoys the same environment in the crystal as in solution. These findings provided us with the first look at a molecular level of the binding of this extensively used fluorescent probe molecule to a protein.

The ANS binding site is near the Cys 1-122 disulfide bridge. When ANS is bound to the crystal of CHT, the only close contact of the neighboring CHT molecule is removed. Then the exterior of the bound ANS is exposed only to solvent. At a larger distance from this bound ANS and on the twofold related CHT neighbor, lies a region of non-polar non-interacting residues. These are Val 235, Val 238, and Leu 242 which rise off the carboxyl terminal helical region of the C chain, and Leu 127 of the B chain. Thus in the crystal, as in solutions of CHT, ANS is bound near the Cys 1-122 disulfide on its protein side and exposed only to solvent on its exterior. This results in the environments and hence lifetimes of bound ANS being the same in the crystal as in solution.

ANS binds with relatively high affinity to CHT in solutions and gives excellent substitution in CHT crystals. Further,ANS

did not affect chymotryptic activity in solution nor perturb CHT structure in the crystal to any appreciable extent. Thus our solution studies are completely compatable with our X-ray crystallographic results.

The main feature of the ANS binding site is that it is by no means what we would call a hydrophobic site. There are several methyl groups contributed by Ala 206, Val 3 and Thr 208 but no aromatics or leucines or isoleucines are found in this region. However, this region does not represent a particularly polar site either. Thr 208 is far to interior in the protein to interact with ANS, its closest approach is by its methyl group which is approximately 5.0 Å removed from the sulfur atom of the sulfonate group of ANS. The disulfide itself, while very polarizable is not particularly polar.

The ANS molecule does lie in a small cleft or depression in the protein structure. This cleft is formed on the top by a loop of the C chain between residues Gly 205-Asn 204-Lys 203 and on the back and side by the Cys 1-122 disulfide and Cys 1, Gly 2, and Val 3 of the A chain.

Many investigators have assumed that ANS binds by primarily hydrophobic interactions and fluoresces only when in a hydrophobic pocket or crevice of the protein. Since the ANS binding site on CHT is not what is generally referred to as a hydrophobic site, such concepts must be revised.

ANS fluoresces intensely when bound to CHT as a consequence of several factors. First, being bound in such a close complex with the protein, it is removed from a totally aqueous environment. While its exterior is still presumably exposed to solvent

molecules, the polar solvent cage that it has while free in aqueous solutions has been greatly reduced. Secondly, the ANS molecule may experience a rather high microviscosity in its binding site. That is, the polar molecules of the protein and solvent near the bound ANS may be restricted from interacting with the ANS molecule during the lifetime of its excited state. This could arise from the geometry of the binding site. Thirdly, the conformation of ANS while bound at this site is such that the naphthalene and anilino rings are nearly planar. Many investigators have suggested that such co-planarity is necessary for intense ANS fluorescence (15,16,17,19). All of these factors may contribute to the intense fluorescence of ANS in low pH solutions of CHT.

The mechanism of binding of ANS to CHT is probably also a composite of several forces. First since ANS can remove some of its bound water by interacting with the protein, some contribution to its binding may come from hydrophobic forces. The contribution from such forces would of course be much less than when, for example, a non-polar molecule is removed from aqueous solution and placed in a truely hydrophobic site. There is also the possibility of ionic interactions of the sulfonate of ANS with the NH_3^+ of the amino terminal Cys 1. These charges are separated by approximately 5.0 Å and could offer some contribution to binding. Hydrogen bond formation between the NH group of ANS and the electrons of the disulfide bridge may also occur since disulfides are quite basic. However, if intramolecular hydrogen bonding is occuring between the NH and SO_3^- of ANS (as in ANS crystals) then this mechanism of binding would be unlikely.

A third mechanism of binding remains. This involves ion-induced dipole forces. The negatively charged sulfonate group of ANS lies very close (within 3.0 Å) of the disulfide bridge. Such disulfide linkages exhibit a resonance character similar to an S=S double bond and probably have π electronic orbitals (128). This makes the disulfide extremely polarizable. The negative charge of the sulfonate of ANS could induce a dipole in the disulfide bridge. This would make the end of the bridge nearest the sulfonate (near Cys 1) more positive relative to the end of the bridge near Cys 122, which would be more negative. This ion-induced dipole interaction could serve to bind the ANS at the disulfide bridge. However, the Cys 122 region being more negative, might exhibit repulsive forces for the π electrons of the lower naphthalene ring (ring 1) which it lies nearest to. This could be the reason for the pucker in the ANS electron density : the π electrons of the naphthalene ring nearest the Cys 122 bend slightly out of the plane formed by the naphthalene ring (ring 2) and the anilino ring. While this mechanism seems more plausable as an explanation of ANS binding, all or any of these (or other) forces could be involved to some degree in the binding of ANS to CHT at this site.

Difference electron density maps of CHT at various pH values minus native CHT (pH 3.6) have allowed Tulinsky and his co-workers to characterize a number of pH conformers of CHT. Examination of such a difference map for pH 7.3 CHT shows a structural change in the ANS binding site relative to its pH 3.6 conformation. This change is in the region of the Cys 1-122 disulfide and the Cys 1 residue. Changes in electron density occur which indicate

a movement of the disulfide up (to lesser y), towards the Lys 203 sidechain (to lesser x) and more exterior to the protein molecule itself (to lesser z). These changes are not dramatic and do not represent a large movement of the disulfide or Cys 1, however, they occur at a level of significance for over nearly 3.0 Å . Changes in the position of cysteine 1 are also observed, especially near the amino terminal NH_3^+ .

Such conformation changes in the region of the disulfide bridge could quench ANS fluorescence by several mechanisms. First, this conformation change could push the bound ANS molecule away from the protein. Being more removed from its proximity to the protein structure would allow it to become more solvated. Greater interaction of polar solvent molecules with this more "exposed" ANS would reduce its fluorescence yield and lifetime and red shift its emission wavelength maxima. Secondly, this conformation change could bring the ANS closer to chemical quenching groups on the protein, such as, the carboxyl group of Asn 204 or the NH_3^+ group of Cys 1. Finally, the conformation change could allow the disulfide to form a charge-transfer complex with the ANS molecule. Cysteine is a good electron donor (129,130) and we have observed quenching of ANS fluorescence in alcohol solutions with high concentrations of cysteine. If such a complex formed with one cysteine residue as an electron donor and with ANS as an electron acceptor, the fluorescence of ANS could be quenched quite effectively. Each of these mechanisms could explain the quenching of ANS fluorescence at high pH relative to its intense fluorescence at pH 3.6.

X-ray and fluorescence analysis of CHT-ANS crystals at

pH 6.7 are now being completed. These results should allow us to determine directly what conformation changes occur in ANS and in the ANS binding site at high pH to so dramatically quench ANS fluorescence.

Such detailed information of the changes that occur around the ANS binding site with the conversion of CG to CHT was not available. However, some possible mechanisms of the conformation changes involved in producing the observed fluorescence changes with activation of CG are suggested.

The main step in the activation of CG is the tryptic cleavage of the Arg 15-Ile 16 peptide bond to form π-CHT. This cleavage frees the A chain residues 1-15 and allows them to enjoy quite alot of rotational freedom. This A chain is essentially anchored to the protein by the Cys 1-122 disulfide bridge. This new freedom of movement of the A chain could easily result in changes in the position of the disulfide bridge or in the first few residues of the A chain: Cys 1, Gly 2, and Val 3. Such changes could alter the fluorescence of bound ANS.

Some autolytic event also reduces CG-ANS fluorescence in a similar way as the initial tryptic cleavage. Autolysis of the Leu 13-Ser 14 peptide bond is known to occur and this cleavage would again free the A chain (which would now consist of only 13 residues) so that it might have this greater freedom of movement. Thus both the tryptic cleavage of the Arg 15-Ile 16 peptide bond and the chymotryptic cleavage of the Leu 13-Ser 14 peptide bond could produce similar conformation changes near the Cys 1-122 disulfide region and yield the similar fluorescence changes observed with tryptic activation of CG and chymotryptic

autolysis of CG.

By correlating fluorescence solution studies with X-ray crystallographic studies, we have provided the first high resolution "picture" of the extensively used fluorescent probe molecule, ANS, binding to a protein. We have also shown that this "picture" of ANS binding to the crystal of CHT is representative of ANS binding to CHT in solutions where it is highly fluorescent. Unlike most investigators would have expected, (ourselves included) ANS does not bind in a hydrophobic crevice nor does it appear to bind by hydrophobic forces to any large extent. Its high fluorescence while bound to CHT and its binding to CHT may be explained by other mechanisms. Definite pH dependent conformational changes occur within the ANS binding site which dramatically alter the fluorescence of bound ANS. Speculation on the mechanism of these pH induced fluorescence changes is allowed by comparison of the structure of the protein in the ANS binding site at pH 3.6 and at pH 7.3. However, conclusive interpretations of what specific conformational changes alter its fluorescence properties awaits the pH 6.7 CHT-ANS structural work.

VIII. EXPERIMENTAL

Alpha-chymotrypsin, 3X crystallized and with an activity of 54-61 U/mg (CDI); chymotrypsinogen A, 3X crystallized (CG4A); trypsin, 193 TAME U/mg (TRL7LA), and tosylated CHT (TCDI,6FA) were obtained from Worthington Biochemical Corp. Freehold N. J. and used without further purification. β-phenyl proprionic acid from Fulka, N-formyl-tryptophan from International Chemical and Nuclear Corps, Irvine, Cal.; proflavin (3,6 diamino acridine) from Aldrich Chemical Corp. Mil. Wisc., N-acetyl-L-tyrosine ethyl ester (ATEE) from Sigma Biochemical Corp. St. Louis, Mo., 2-ρ- toluidinylnaphthylene-6-sulfonate (TNS) and 1-anilino-8-naphthalene sulfonate (ANS), the ammonium salt were obtained commercially from Sigma Biochemical.

Absorption spectra were run on a Cary 15 recording spectrophotometer and fluorescence spectra on an Aminco-Keirs spectrophosphorimeter modified with an EMI 978 IR phototube for lower dark current, higher gain, and better red response.

The pH fluorescence curves were prepared in buffers made for each distinct pH value. Below pH 5.0 a walpole's acetate buffer was used. Similar pH-fluorescence curves were obtained in aqueous solutions when the pH was adjusted by titrations with acetic acid.

In the tirtration studies of CHT with ANS, the fluorescence intensities were corrected for self-absorption of the incident light using the relationship $I_c = I_o\ 2.303 \epsilon_{350} F_o / (1-10^{\epsilon 350 F_o})$ where F. is the total dye concentration, $\epsilon 350$ is the molar extinction coefficient of ANS at 350 nm (5000 $M^{-1}cm^{-1}$)

I_c and I_o refer to the corrected and observed intensities of fluorescence respectively. Self-absorption of the fluoresced light was negligible at wavelengths above 430 nm. Absorption of the protein was always less than .1 absorbance units at the wavelength of ANS excitation and emission and therefore would not interfere with the relative fluorescence yields.

Assays of chymotryptic activity were conducted in .067 M phosphate buffer at pH 7.0. The hydrolysis of N-acetyl tyrosine ethyl ester ($5x10^{-4}$) with $2x10^{-7}$M chymotrypsin was followed by the change in absorbance at 237 nm. in the presence and absence of ANS.

The conversion of CG to CHT was conducted at pH 7.0 in .1M phosphate buffer. To standardize the amount of autolysis that might occur, activation was always initiated on twenty minute old solutions of CG.

The nanosecond fluorescence decay curves were obtained with a single photon counting, time resolved spectrophotometer built in our laboratory and described elsewhere (114). This instrument consist mainly of a nonosecond pulser, Ortec 9352, time to amplitude converter, Ortec 475, multichanner analizer, Nuclear Data 1100, a single photon counting phototube, RCA 8850, and a nanosecond flash lamp. Excitation was through a CS 7.51 corning absorption filter and emission was monitored at 475 through a Bosch and Lomb monochrometer. The parallel and perpendicular components of the fluorescence decay curves were obtained using plastic polarizing lenses mounted on the cell holders. The fluorescence lifetimes were determined in .067 M buffers, 10^{-4}M

in protein and $5x10^{-5}$M in ANS by observing the emission at 475. Depolarization studies were conducted under these same conditions. Deconvolution of the lamp and data curves were performed using a deconvolution analysis developed by Ware (115) and the data were fit by a least squares analysis through the Michigan State University chem fit and computing facilities.

Crystals of CHT were grown and the X-ray crystallographic structural studies of CHT-ANS were conducted as described previously for CHT and CHT inhibitors (43,96,105,106). Native CHT crystals were layered with a saturated solution of their soaking solution (75% saturated ammonium sulfate at pH 3.6) by replacing small increments (.5 ml) of soaking solution with an equal volume of soaking solution plus ANS. After equilibrating for one month these crystals (which were observed to be highly fluorescent) were mounted in capillary tubes and a three-dimensional , 2.8 Å resolution difference electron density map of CHT-ANS minus native CHT was prepared. Changes in the X-ray absorption characteristics of the CHT-ANS crystals were observed after additional soaking. Three month old soaking crystals were found to be fully equilibrated and a three dimensional analysis of these crystals was conducted. The only difference between the two crystals was the greater substitution of ANS at the same site in the crystals that were fully equilibrated.

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