A BIO-OPTICAL PROBE OF TRYPTOPHYL EXPOSURE

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ABSTRACT

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By

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The charge-transfer complex formed between FMN (flavin mononucleotide) and tryptophan or FMN and the "exposed" tryptophyl residues of proteins in solution, provides an excellent Bio-optical probe of tryptophyl exposure. In such complexes an electron is transfered from the indole ring of tryptophan to the flavin producing and stabilizing the flavin semiquinone. Characteristic of such complex formation is a difference absorption maximum near 500nm. This absorption is characteristic of the flavin semiquinone and indicates that complex formation and electron transfer has occured. Since such complex formation is highly specific for tryptophan among amino acids and can be easily monitored by difference absorption spectroscopy we were able to devise a convienent and reliable technique for determining tryptophyl exposure in proteins. We successfully applied this technique to several proteins in their native and heat denatured states.

In addition to the new absorption and difference absorption maximum at 500nm, such complex formation also results in a decrease in the molar extinction coefficients of both the 370nm and 450nm flavin absorption bands, a decrease in the oxidation-reduction potential of the flavin, and a complete quenching of both flavin and tryptophan fluorescence by the formation of a non-fluorescent charge-transfer complex.

These same phenomena are observed in FMN-TRP solutions, flavinylpeptides of tryptophan, and several flavodoxin proteins upon flavin binding. This lead us to suggest that similar complex formation occurs in each case.

Several studies have implicated a tryptophyl residue in close association with the flavin moiety in the FMN binding site of several flavodoxins. This has very recently been verified by high resolution X-ray crystallography. The evidence that we present and such X-ray analysis provides the first conclusive evidence that such a charge-transfer complex occurs in any flavoprotein.

Since the flavodoxins serve as electron carriers to and from other proteins, with the flavin serving as the electron collection point, the knowledge that such specific complex formation occurs in these proteins should prove to be of great importance in advancing the understanding of the mechanism of flavoprotein action.

A BIO-OPTICAL PROBE OF TRYPTOPHYL EXPOSURE

By

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TO MY MOTHER AND IN MEMORY OF MY FATHER

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INTRODUCTION

Proteins have been found to be directly involved in every known biological process except for the storage of genetic information. Changes in the conformation or three dimensional structure of proteins may have serious consequences on their biological activity. Direct relationships between protein structure and function are invaluable in the sense that they allow us to more fully understand the mechanism by which proteins function.

Tryptophan is one of the twenty basic amino acid building blocks of proteins. It is present in most proteins. Tryptophan is distinct among amino acids in the sense that it is the most hydrophobic of all amino acids (123). Perhaps it is because of its hydrophobicity that it is often found in hydrophobic active sites or other binding sites and crevices in the protein structure. It may be required at these sites to provide the correct environment for protein-substrate or protein-protein interactions and to aid in establishing the specificity of such interactions.

The conformational changes in protein structure which are of interest are those that alter the function of the protein. Since these changes most often involve changes in the active site of secondary binding sites, they often involve changes in the exposure or the tryptophyl residues in these sites. Thus changes in tryptophyl exposure can often provide a convenient handle for conformational changes in proteins, protein-protein and protein-substrate interactions.

Tryptophan also enjoys the position as the main chromophoric group of proteins. Both its absorption and emission are more intense and are resolvable from the absorption and emission of the other amino acid

chromophores, tyrosine and phenylalanine. The sensitivity of the absorption spectra of tryptophan to its environment has made techniques such as ultra-violet difference spectroscopy and solvent perturbation difference spectroscopy useful in determining the extent of tryptophyl exposure.

Chemical reactivity studies involve the reaction of a chemical reagent with the exposed tryptophyl residues of the protein. A complex is formed between the reagent and the exposed tryptophyl residues which can be analyzed and quantitated either spectrally or by subsequent amino acid analysis.

Individual techniques prove to be somewhat less than adequate for monitoring tryptophyl exposure and only when used together can they provide an unambiguous picture of tryptophyl exposure.

X-ray crystallographic techniques, of course, offer the ultimate in protein structure determination. Inhibitive of its use is the tremendous expense in time and effort required for complete structural determination and the fact that the protein must be able to form crystals which are stable over long periods of time and under intense irradiation. Crystallographic analysis is also limited in the sense that it offers only a static picture of the protein structure and thus the dynamics of protein interactions are often lost. In the long run, however, the results of any technique for determining protein structure must agree with the X-ray determined structure or have differences that are explainable in terms of the differences of protein structure in the crystal and in solution.

Tryptophan is also distinguished among amino acids in the sense that it is the most efficient electron donor of all the amino acids.

The role that it plays as an electron donor in proteins is far from clear. FMN (flavin mononucleotide or riboflavin-5'-phosphate), which is known to be an excellent electron acceptor, forms a red complex when in solution with tryptophan. This red color appears as a broadening or a red shoulder in the 450nm flavin absorption band and appears clearly as a difference absorption maxima near 500nm. This spectra is characteristic of the partially reduced flavin (i.e., flavin after it has accepted an electron) and indicates that charge transfer has occured. The flavin semiquinone is stabilized by complex formation with tryptophan. Both the red color and the 500nm difference absorption band observed in solutions of flavin and tryptophan is highly specific for tryptophan among amino acids.

In FMN-protein solutions complex formation occurs between the flavin and the exposed tryptophyl residues of the protein. Complex formation is easily monitored spectrophotometrically by the absorbance at the 500nm difference absorption peak. We have found that such complex formation provides a convenient and reliable means of determining tryptophyl exposure in proteins. The technique that we describe allowed us to successfully determine the number of exposed tryptophyl residues in several native and denatured proteins.

FMN-tryptophan complexes are seen to be non-fluorescent and acts as an energy sink. Complex formation thus serves to quench both tryptophan and flavin fluorescence. In FMN-tryptophan solutions, flavinylpeptides of tryptophan, and several flavodoxin proteins, similar absorption spectra, difference absorption spectra, mutual quenching of flavin and tryptophan fluorescence, and decreases in the oxidation-reduction potential of the flavin implies that similar complex formation may occur

in each. This is especially interesting since these flavodoxins, which function as electron carriers to and from other proteins, have FMN as their only prosthetic group. This evidence leads us to suggest that a complex is formed between FMN and a tryptophyl residue in the FMN binding site of some flavodoxins. In this complex the flavin lies in close proximity to a tryptophyl residue and an electron is transfered from the indole ring of tryptophan to the isoallazine ring of the flavin. Recent X-ray analysis of two flavodoxins confirm the existence of a tryptophyl residue in the FMN binding site in very close proximity to the flavin moiety. This is accepted as further evidence of our suggestion that such complex formation occurs in some flavodoxins.

We provide the first strong evidence that such a charge-transfer complex occurs in any flavoprotein. The existence of such a specific interaction in these flavodoxins, which function in electron storage and transport, should provide a clearer understanding of the mechanism of flavoprotein action.

Chemical Reactivity of Tryptophyl Residues

One of the most widely applied methods of exposure analysis is chemical modification of tryptophyl residues of proteins. In these techniques the reactivity of tryptophyl residues with specific chemical reagents is observed. Reagents are desired that will react specifically with the exposed tryptophyl residue without perturbing the structure of the protein and allow a calculation of the number of reacting or exposed residues of the native protein. Most reagents, however, tend to be far from ideal either in their degree of perturbation of the system, or in their lack of specificity for tryptophyl residues.

N-Bromosuccinimide (NBS)

NBS is often used to oxidize the tryptophyl residues of proteins. The reaction is not specific for tryptophyl residues. Tyrosine, histidine, methionine, cysteine, cystine, and lysine are also oxidized (1). Most oxidations with NBS are done at pH three to four. The reactivity of NBS with tryptophan is found to be the greatest in this pH range and it decreases substantially as the pH is raised to seven. The extent of oxidation is often determined by measuring the decrease in absorbance at 278nm of the oxidized protein solution (1). The marked dependency of reactivity with pH is thought to result from the spectral procedure involved in this technique (2).

Kronman and Robbins (2) have monitored the reaction of NBS with lysozyme spectrally and with subsequent amino acid analysis. They found that this reagent was not specific for tryptophyl residues. Tyrosine and histidine residues were destroyed, even at concentrations of NBS so low that its reaction with tryptophyl residues was incomplete. Very seldom do investigators monitor the reaction of such chemical reagents

with amino acid analysis or any other physicochemical technique and thus the specificity of the reaction is often falsely assumed.

At acid pH, NBS oxidation results in the hydrolytic cleavage of the polypeptide chain of the protein (4). This may alter the conformation of the native enzyme and perhaps expose additional tryptophyl residues. Oxidation at pH five to seven can be carried out without breakage of the polypeptide chain (3). This approach would seem to be more advantageous since it eliminates the possibility of exposing additional residues as a consequence of polypeptide chain cleavage. Perturbation of the native structure of the enzyme and the lack of specificity of NBS for tryptophyl residues are seen to be the two major disadvantages of such chemical oxidation studies to determine tryptophyl exposure.

Kronman and Robbins (4) point out many of the pitfalls of this reagent and many examples of its application to specific proteins. Witkop (1), who developed the spectral titration technique, presents a more complete review and detailed description of this method.

2-hydroxy-5-Nitrobenzyl Bromide (HNBB)

This reagent has been used by Koshland et al. (5, 6, 7) for the modification of tryptophyl residues of proteins. In this reaction a derivative of HNBB is left attached to the exposed tryptophyl residues of the protein to form a HNB-tryptophan complex. The number of such labeled tryptophyl residues is generally determined by the absorbancy of these complexes on the protein at 412nm (5). Although HNBB is more specific for tryptophan at acid pH, cysteine and tyrosine residues will react and other techniques, such as amino acid analysis, should be utilized to determine the actual specificity of the reaction. Another difficulty with HNBB oxidation is the possibility of two HNBB molecules

reacting with a single tryptophyl residue to produce a di-substituted residue and the consequent errors in exposure determinations.

Kronman and Robbins (4) present many examples of the use of this reagent in various protein systems. Since HNBB is useful over a wide pH range (pH2 to pH7) and is seemingly more specific for tryptophyl residues than NBS, it is perhaps a better reagent, though it has enjoyed less use to date. A water soluble derivative of this reagent has also been used in determinations of tryptophyl exposure.

Hydrogen Peroxide

Hachimori et al. (9) have found the reagent hydrogen peroxide at pH 8.1 to 9.4 in .5M bicarbonate buffer with 10% dioxane, a suitable reagent for analysis of tryptophyl exposure. The reaction of this reagent with tryptophan can be followed as the decrease in absorbance at 282nm using a difference molar extinction coefficient of 3.49×10^{-3} for the oxidation of tryptophan.

The absence of significant spectral changes in the absorption of tyrosine and phenylalanine indicate that minimal reactivity is exhibited towards these residues. Although spectral changes in histidine absorption occur upon addition of this reagent, no further evidence indicating reactivity of histidine residues with hydrogen peroxide has been found. Other amino acids show no change in absorption near 282nm when treated with this reagent. Reaction with residues other than tryptophan may occur and coupling of this technique with other methods of monitoring reactivity of hydrogen peroxide such as subsequent amino acid analysis is a necessary but seldom used precaution.

Kronman and Robbins review the application and limitations of this reagent (4). They point out that the reactivity of this reagent with

tryptophan is dependent on pH, the dioxane concentration, and the type of buffer ion used.

Such chemical reagents as these have been used extensively to determine the degree of tryptophyl exposure and in many cases the total number of tryptophyl residues in the denatured protein. A measure of their reactivity is generally obtained by either spectral techniques or by amino acid analysis for the reactive residues. The most serious drawbacks of such chemical reactants is their lack of specificity for tryptophyl residues and the conditions such as pH, which are required for the reaction to occur properly. The fact that these reagents may seriously perturb the native structure of the protein as they react with its tryptophyl residues presents yet another serious limitation. In many instances where these reagents have been used, no determination of the reactivity as a function of reagent concentration was made and it is thus uncertain whether the maximal reaction occured or not.

Such chemical reagents are useful but their results should only be trusted when coupled and complemented by other methods of tryptophyl analysis such as X-ray data or other spectral techniques including ultra-violet difference absorption spectra and solvent perturbation. These spectral techniques for tryptophan exposure analysis can best be understood only after we have some understanding of tryptophan absorption in general.

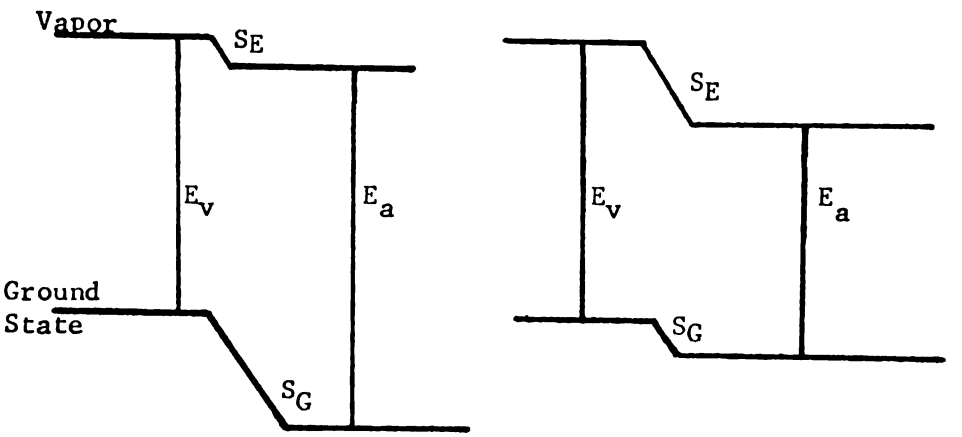
Molecular Absorption

The act of absorption is an electronic process which occurs on the order of 10^{-14} 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. Thus relaxation of the solvent molecules takes place in times of the order of 10^{-11} seconds at room temperature to allow a reorientation of the solvent molecules to reach an equilibrium configuration compatible with the excited state dipole moment of the solute.

If such solute-solvent interactions can serve to stabilize the excited state more than the ground state during the act of absorption, then the absorption band corresponding to the transition between these two states will be red shifted to lower energies, relative to absorption in the vapor. If, however, such solute-solvent interactions stabilize the ground state more than the excited state during absorption, the absorption will be blue shifted to higher energies. Both of these cases of solvent effects on a polar solute molecule are illustrated in Figure I.

Now we wish to consider some of the characteristics of tryptophan absorption, the types of molecular interactions and solvent effects that alter its absorption spectra in solution, and how environmental conditions may influence tryptophyl absorption in proteins.



Case a Case b

Figure I. Solvent effects on Absorption

E_v, E_a, and E_b represent the energy of the absorption in vapor, case a and case b respectively. S_E and S_G represent the solvation energies of the excited and ground states respectively.

Case a. $S_E < S_G$

Solvent interaction lowers the ground state more than the excited state. Absorption is blue shifted; $E_a > E_v$.

Case b. $S_E > S_G$

Solvent interaction lowers the excited state more than the ground state. Absorption is red shifted; $E_b < E_v$.

Tryptophan Absorption

Solvent effects on the absorption spectra of tryptophan and closely related indole derivatives have been widely investigated. Such studies allow one to obtain information regarding the environment of tryptophyl residues in proteins and to follow changes in that environment that result from conformational changes in proteins.

In view of the poor solubility of tryptophan in nonpolar solvents, absorption studies in such solvents are generally performed on indole and indole derivatives which have the same mobile electronic system as tryptophan. Figure II represents the absorption spectra of indole in aqueous solution and in hexane. The absorption spectra exhibits more vibrational structure in tne nonpolar solvent compared to water. In hexane, absorption spectra maxima are clearly observable at 261, 266, 279, and 287nm with shoulders at 271 and 276 nm.

Cheinitski and Konev (10) observed the gradual transformation of the spectra of indole in hexane solutions as n-butanol was added. Their results indicate that two species of indole molecules were present. One form resulted from hydrogen bonding of the alcohol with the indole NH group and the other form, presumably, from solute-solvent interactions other than hydrogen bonding (11). All of the absorption maxima were shifted to the red (longer wavelength).

An interesting feature accompanying this addition of alcohol was the fact that different peaks undergo shifts to various extents. The short wavelength maxima at 276nm undergo a red shift of about 700cm^{-1}, while the longer wavelength maxima at 279 and 287nm are shifted by only 150 cm^{-1}. These unequal shifts due to hydrogen bonding imply that the long wavelength absorption band of tryptophan consists of two overlapping ¶*←¶ electronic transitions. These have been defined as the

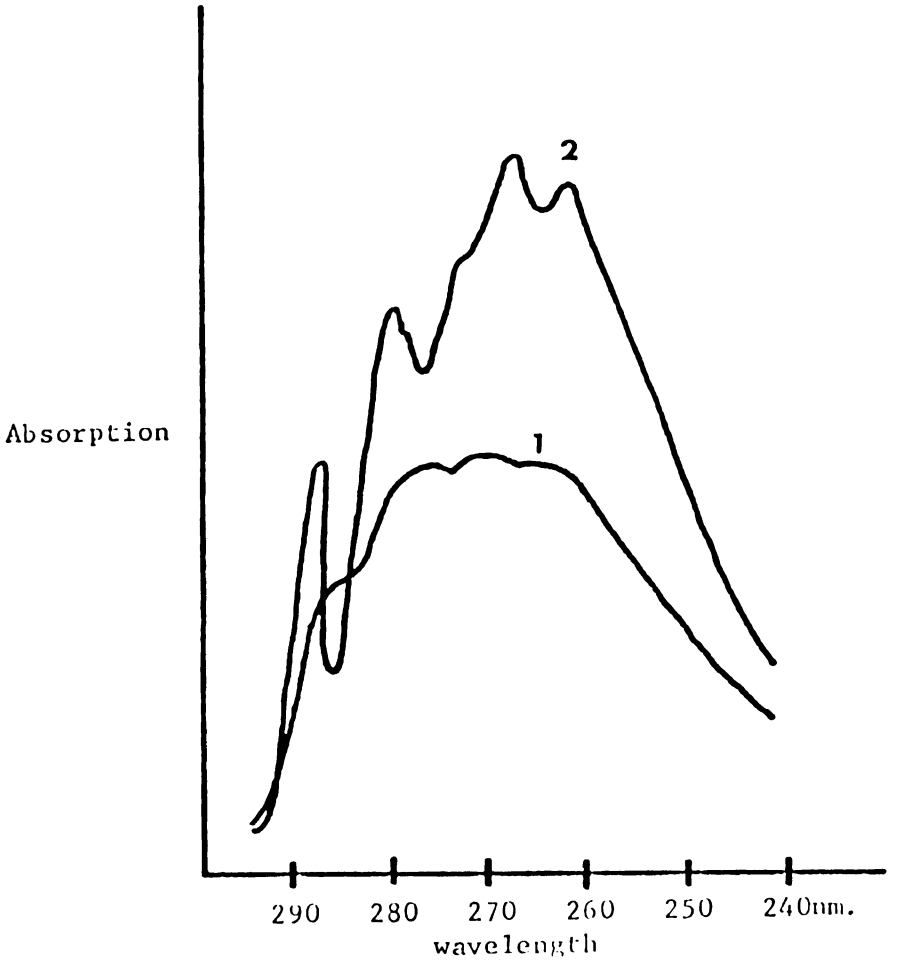


Figure II. Absorption Spectra of Indole
1, Indole in a water solvent.
2, Indole in an n-hexane solvent.

$^1L_b \leftarrow {}^1A$, with vibrational maxima at 279nm and 287nm separated by 1000 cm^{-1} and the $^1L_a \leftarrow {}^1A$ which occurs at shorter wavelengths with vibrational spacing of 700 cm^{-1}.

Fluorescence polarization excitation spectra by Konev et al (12, 13, 14) with tryptophan, showed that tryptophan absorption is a superposition of two transitions, the ^1L_a and the ^1L_b, whose oscillators are nearly at right angles to one another. Fluorescence polarization excitation spectra of indole by Song and Kurtin (15) gave similar results, with the ^1L_b oscillator oriented along the long axis of the molecule while the ^1L_a oscillator is oriented along the short axis of the molecule and passing through the nitrogen of the indole as shown in Figure III. From these observations we might expect that substitution at the nitrogen would have a large effect on the ^1L_a absorption.

Electron density calculations (16) on indole shows that the first two excited singlet states of indole have a much larger permanent dipole moment than in the ground state, the ^1L_a being larger than the ^1L_b. Upon excitation, the charge density is decreased on the nitrogen atom. This decrease is larger for the ^1L_a state than for the ^1L_b. It follows that the acidity of the NH group of indole follows the order $^1L_a > {}^1L_b >$ ground state. Thus the energy of hydrogen bonding is greater in the ^1L_a state than the ^1L_b state or the ground state, leading to a much larger red shift of the ^1L_a transition compared to the ^1L_b transition in hydrogen bonding solvents.

Very pronounced shifts in the ^1L_a absorption are also observed as a result of substitutions at the pyrrolic nitrogen atom (17, 18). These effects are consistent with a ^1L_a oscillator which is oriented along the short axis of the molecule. Substituents and hydrogen bonding have com-

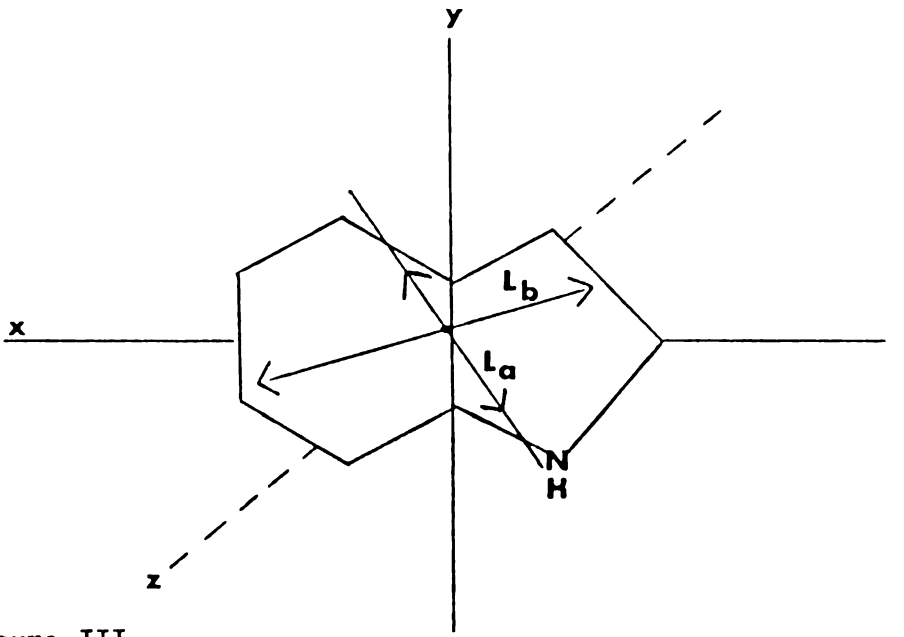


Figure III.
Polarization of Singlet-Singlet π -π^* transition in Indole.

Polarization of L_b is along the X axis

Polarization of L_a is along the Y axis

The calculated mutual orientation angle between the ^1L_a and the ^1L_b is 78^o.

paratively little effect on the ^1L_b transition which is oriented in the plane of the molecule also but along the long axis (19, 20).

Konev has suggested that the absence of absorption-fluorescence mirror symmetry in indole is further evidence of the composite nature of the first absorption band (21). He has shown that each of these $\pi^* \leftarrow \pi$ transitions are polarized in the plane of the indole ring but because the transitions overlap so extensively in the near ultraviolet it is difficult to separate tryptophyl absorption into its ^1L_a and ^1L_b components.

Strickland et al (22) have used the fact that the position of the ^1L_a transition is more sensitive to solvent perturbation than the position of the ^1L_b transition to unscramble these transitions. 3-methylindole in perfluorinated hexane has its $0\text{-}0\,^1L_a$ at 285.2nm with vibronic bands at $0 + 1700\ cm^{-1}$, $0 + 245\ cm^{-1}$, and the $0\text{-}0\,^1L_b$ band at 288nm with vibronic bands at $0 + 730\ cm^{-1}$, $0 + 980\ cm^{-1}$, $0 - 760\ cm^{-1}$. The remaining higher energy ^1L_b bands were unresolvable.

In nonpolar solvents both the $0\text{-}0\,^1L_a$ and $0\text{-}0\,^1L_b$ transitions occur at nearly the same positions, (i.e.), at 289nm in perfluoromethylcyclohexane (23). More polar, more interacting solvents, cause a loss of structure in the absorption spectra and cause selective red shifts in the ^1L_a band and to a lesser extend the ^1L_b band. It is important to consider how solvent properties such as hydrogen bonding ability, polarizability, and dipole moment can influence the absorption spectra of the indole ring.

Hydrogen bonding.

Two main types of hydrogen bonding seem to be involved in indole-solvent interactions. The first type involves the formation of a

hydrogen bond involving the proton of the pyrrolic nitrogen atom and an appropriate proton accepting group of the solvent. In a water solvent the oxygen atom serves as the proton acceptor and this type of hydrogen bonding, type I, is denoted by (NH...O).

The second type of hydrogen bonding, type II, involves a hydrogen bond through the pyrrolic nitrogen atom of indole and a proton donated by the aqueous solvent molecule. This type of hydrogen bonding is denoted by (HN....H). Both type I and type II hydrogen bonding between indole and water are illustrated in figure IV.

Hydrogen bonding via the proton of the pyrrolic nitrogen atom (type I) seems to cause a red shift mainly in the ^1L_a absorption band. While the ^1L_a band undergoes a red shift of 1300 cm^{-1}, the ^1L_b band sees only a 61-185 cm^{-1} red shift as a result of such hydrogen bonding (23). The size of the red shift in the ^1L_a band seems to increase in the same order as the equilibrium constant for hydrogen bonding through the NH group (19, 24, 25). The strongest hydrogen bonds seem to give the largest red shifts of the ^1L_a.

Hydrogen bonding of the pyrrolic nitrogen with the proton of water solvents (type II) has quite a different effect. Since the charge density of this nitrogen atom decreases in the excited state a weaker hydrogen bond will be formed with the proton of the solvent and thus the ^1L_a and to a lesser extent the ^1L_b peaks will be blue shifted due to this type of hydrogen bonding. Li (8) has estimated the blue shift due to this type of hydrogen bonding to be 322 cm^{-1} for the ^1L_b and 706 cm^{-1} for the ^1L_a in water relative to a 3-methylpentane solvent. Hydrogen bonding of the proton of the pyrrolic nitrogen to water: (NH....O) involves only a 24 cm^{-1} red shift of the ^1L_b and a 186 cm^{-1}

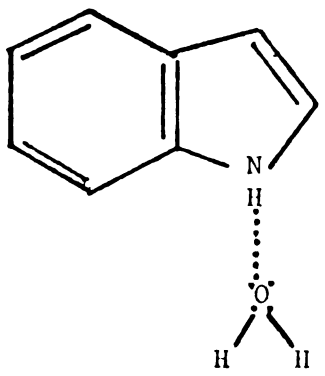
red shift of the ^1L_a band relative to this solvent.

Thus we see that in water solvents relative to non-hydrogen bonding solvents the red shifts in both the ^1L_a and ^1L_b due to hydrogen bonding of type I, NH....O, are small relative to the blue shifts in both the ^1L_a and ^1L_b which result from hydrogen bonding of the pyrrolic nitrogen atom to the water solvent, type II, HN....H.

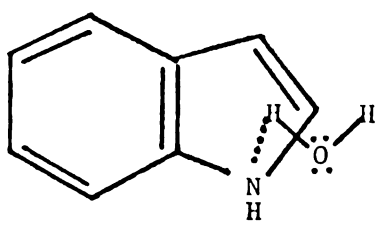
Polarizability

The polarizability of the medium is also known to affect the position of the tryptophyl absorption bands. Yanari and Bovey (27) have examined the spectra of benzene, phenol, and indole as model compounds for the side chains of phenylalanine, tyrosine, and tryptophan, respectively, as a function of solvent polarizability. In nonpolar solvents they observed that the spectra of each was shifted to the red with increasing refractive index (increasing polarizability) of the solvents. Apparently substantial red shifts can occur as a result of the altered polarizability of the medium alone.

The interactions between tryptophan and a highly polarizable medium are due mainly to dipole-induced dipole forces. In an isotropically polarizable medium, such interactions are independent of the orientation of the solvent molecules. Since the dipole moment of the ^1L_a excited state of indole is 7.3D and only 2.3D in the ground state (30), the energy of the excited state will be lowered more than that of the ground state, resulting in a red shift of the absorption spectrum. The magnitude of the shift will be greater for the ^1L_a compared to the ^1L_b band, reflecting the larger increase in the dipole moment as a result of excitation to the ^1L_a state.



Type I.



Type II.

Figure IV. Hydrogen bonding between Indole and Water Solvent.

Polar groups

The proximity of polar groups of the solvent represent another factor influencing the position of the tryptophyl absorption bands. The interactions between tryptophan and polar solvent molecules are due to dipole-dipole forces. Suzuki (28) has suggested that a red shift in absorption would be expected if the polar solvent molecules exhibit an attractive interaction with the ground state of the indole ring. In this case the ^1L_a excited state may attract the polar solvent molecules more strongly than the ^1L_b state and much more strongly than the ground state (reflecting the relative magnitudes of the dipole moments of these excited states) resulting in a red shift of both the ^1L_a and ^1L_b transitions.

It is important here, to note, that although the dipole moment of indole and its methylindole derivatives is more than three times greater in the ^1L_a state than in the ground state, the dipole moment of this excited state is shifted approximately 50 degrees from its orientation in the ground state (30). Thus the energy of the excited state may not be lowered efficiently since the absorption will occur to an excited state with a solvent configuration that remains essentially the same as in the ground state. The ground state may be lowered more than the Franck-Condon excited state in polar solvents, resulting in a blue shift in absorption compared to absorption in the vapor or in hydrocarbon solvent. Thus depending on how efficiently the interaction of the polar solvent molecules can lower the energy of the excited state indole ring the absorption will be either blue or red shifted.

Strickland et al (23) have found some evidence that at least the ^1L_a band may be shifted to the red due to interactions of the indole ring

with polar solvent molecules. The ^1L_a absorption of both 1-methyl and 1,2-dimethylindole are red shifted in going from a saturated hydrocarbon solvent to a water solution. Because of the large broadening of the absorption spectra in polar solvents, the exact red shift is difficult to measure, but has been estimated to be at least 423 cm^{-1} for the ^1L_a band for these indoles in going from 2-methylheptane to water.

It is important to note that no hydrogen bonding with water is possible in these methylated compounds and thus hydrogen bonding cannot explain the observed red shift. The altered polarizability of the medium also fails to explain this red shift since a blue shift would be expected in going from a more polarizable to a less polarizable medium. The observed red shift has therefore been explained in terms of dipole-dipole interactions where the polar water molecules are oriented around the polar NCH_3 group or about the entire indole ring. Such oriented water molecules surrounding the molecule must lower the energy of the excited state to a larger extent than the energy of the ground state giving rise to the observed red shift of the ^1L_a absorption band.

An interesting study has been conducted to analyze the magnitude of different solvent effects on the ^1L_a and ^1L_b absorption bands of indole (8). The magnitude of the shift in absorption due to each solvent effect was analyzed relative to the shifts involved in going from 3-methylpentane to water solvents. Their experimental results are presented in table I. From this data we see that dipole-dipole interactions are responsible for large red shifts in the ^1L_a (1009 cm^{-1}) and smaller red shifts in the ^1L_b (176 cm^{-1}). Hydrogen bonding of type I (NH....0) causes a 186 cm^{-1} red shift in the ^1L_a and only a 24 cm^{-1} red shift in the ^1L_b. Hydrogen bonding of type II (HN....H) has rather dramatic

Table 1. Contributions of Dipole-Dipole Interactions and Hydrogen Bonding Interactions to the Observed Absorption Spectral Shifts for Indole.

State	Interaction	H_2O
^1L_b	dipole-dipole	-176
	NH.....O	- 24
	HN.....H	+322
^1L_a	dipole-dipole	-1009
	NH.....O	- 186
	HN.....H	+ 706

+ , indicates the degree of blue shift in cm^{-1} to higher energy.

-, indicates the degree of red shift in cm^{-1} to lower energy.

Taken from Table 1 of reference 8.

effects, producing a 322 cm^{-1} blue shift in the ^1L_b transition and a 706 cm^{-1} blue shift in the ^1L_a. The overall results of these studies suggest that in going from a saturated hydrocarbon solvent to aqueous solution the ^1L_a band will undergo a substantial red shift while the ^1L_b may be slightly blue shifted to higher energy. Their study also indicates that the ^1L_a and ^1L_b are red shifted 897 cm^{-1} and 510 cm^{-1} respectively in hydrocarbon solvents as a result of dispersion and solute dipole-induced dipole interactions relative to the vapor spectra.

Thus, in conclusion, we see that the polarizability, dipole moment and hydrogen bonding ability of the solvent all serve to influence the position of the tryptophyl absorption spectra which is a composite of two overlapping transitions, the ^1L_a and the ^1L_b.

While shifts of both the ^1L_a and ^1L_b are generally in the same direction the ^1L_a is usually more affected than the ^1L_b and experiences larger shifts. The polarizability of the medium seems to be quite important in determining the position of the absorption maxima. Decreases in the polarizability of the solvent are accompanied by blue shifts. More polar solvents may serve to slightly red shift tryptophyl absorption. Hydrogen bond formation between the proton on the pyrrolic nitrogen atom of indole and the appropriate proton acceptor of the solvent is responsible for red shifts while hydrogen bond formation between this nitrogen atom and the appropriate proton donating solvent is responsible for somewhat larger blue shifts in absorption.

Tryptophyl Absorption in Proteins

In proteins we are concerned with residues in two very general environments (24), those that are "buried" and lie in the interior of the protein in a nonpolar but very polarizable environment and those that are "exposed" to the more polar but less polarizable water environment. Hydrogen bonding may occur in each environment, in one case with solvent molecules and in the other case with specific groups of the protein.

The blue shifts of the absorption of phenylalanine, tyrosine, and tryptophan that are observed as many proteins are structurally modified (e.g. partially denatured) exposing additional aromatic side chains to the solvent are closely paralleled by changes in the spectra of their respective model compounds (benzene, phenol and indole) as they are transfered from solvents of higher refractive index (hydrocarbon solvents) to solvents of lower refractive index (aqueous solvents). Thus many of the spectral changes that are observed in proteins are compatable with the picture of aromatic residues in a highly polarizable protein interior that upon denaturation are exposed to a less polarizable environment.

In the nonpolar yet more polarizable protein interior a variety of interactions may occur. Because the interior of the protein is more polarizable, the energy of the absorption will be lowered and a red shifted absorption will result relative to a tryptophan molecule in a less polarizable aqueous environment. Because of the nonpolar nature of the protein interior we would expect less interaction of polar groups with interior residues. However, if a polar group is nearby it could serve to red shift the tryptophyl absorption band.

Hydrogen bonding of the pyrrolic nitrogen atom with the appropriate

proton acceptor group (e.g. NH....O) might occur in many regions of the protein interior. The strength of the hydrogen bonds that form with tryptophyl rings determines the degree of the red shift in their absorption. Since the red shifts are so large for certain hydrogen bonds, they may have a very large influence upon the position of the ^1L_a absorption even for indole rings partially exposed to the surface of buried in slightly polar regions of the protein.

Strickland et al (23) summarize the shifts of the ^1L_a band which are expected to occur as the tryptophyl NH group hydrogen bonds to various proton acceptors in the protein. They conclude that in proteins the ^1L_a band may be red shifted 1356 cm^{-1} due to hydrogen bonding. The largest red shifts are anticipated for hydrogen bonding to the $-\ddot{N}=$ of the histidyl side chains, the carbonyl oxygen of the peptide backbone and of side chain amides and carboxylate ions.

Reiniche et al (32) point out that in indole aromatic interactions, the ^1L_a band may be shifted as a result of weak hydrogen bonding of the NH group of indole with the π orbitals of neighboring aromatic rings of phenylalanine, tyrosine, histidine or other tryptophyl residues. This effect may be of even more significance since recently several proteins have been found to have "pockets" of aromatic amino acids in their three dimensional structure (33, 34). The interactions of tryptophyl residues with aromatic rings in these aromatic pockets may induce red shifts in absorption both by this weak hydrogen bonding and also by the fact that aromatic rings are more polarizable than aliphatic side chains.

Hydrogen bonding of the pyrrolic nitrogen atom to an appropriate proton donor (HN....H) seems to be very much favored in water solvents and probably occurs to a very limited extent in the protein interior.

The exposed tryptophyl residue lies in a less polarizable aqueous environment. The decreased polarizability of its environment would be expected to blue shift its absorption relative to a buried residue. However in a water solvent the absorption spectra of indole and indole derivatives are probably the result of several factors other than altered polarizability alone, since the ^1L_a band is red shifted and the ^1L_b band is blue shifted relative to their position in saturated hydrocarbon solvents. The ^1L_b band which is not significantly affected by hydrogen bonding or dipole-dipole interactions may be blue shifted as a consequence of the decreased polarizability of aqueous solvents. The ^1L_a is much more sensitive to hydrogen bonding and dipole-dipole interactions and they may serve to red shift the absorption band corresponding to this transition.

Strickland et al (23) have suggested that hydrogen bonding of the pyrrolic nitrogen atom to a water solvent, type I: (NH....O) contributes to the red shift of the ^1L_a band. Hydrogen bonding of the pyrrolic nitrogen to water of type II: (HN....H) serves to even more extensively blue shift the ^1L_a absorption. Thus when both types of hydrogen bonding are considered, a blue shift in the ^1L_a would be expected to occur.

Dipole-dipole or electrostatic interactions of water molecules oriented around the polar NH group or about the entire indole ring may also serve to selectively red shift the ^1L_a absorption in water solvents.

In conclusion we see that the absorption of buried residues may be red shifted as a consequence of the higher polarizability of the protein interior, hydrogen bonding to the appropriate proton acceptor group within the protein, and perhaps to a lesser extent due to nearby polar

groups in the protein interior. While dipole-dipole interaction may serve to red shift the absorption of solvent exposed residues, the lower polarizability of the aqueous solvent and hydrogen bonding of the exposed residue to water will both serve to blue shift the absorption of the exposed residue. These considerations lead to the conclusion that buried residues lying in the nonpolar but highly polarizable protein interior of the protein should exhibit a red shifted absorption relative to residues which are exposed to the aqueous environment surrounding the protein in solution. The observation that the denaturation of most proteins is followed by blue shifts in their tryptophyl residues supports this conclusion.

U.V. Difference Spectroscopy and Solvent Perturbation

It is tempting at first glance to correlate chromophores with red shifted absorptions with those residues that are "buried" in the more polarizable and less polar interior of the protein and those chromophores with blue shifted absorption spectra with residues, that are "exposed" to the aqueous water environment as the result of some perturbation. However, several factors and not the polarizability of the medium alone are involved and each may influence the position of the absorption bands. Hydrogen bonding both with solvent and with groups in the protein must be examined as well as the interaction of the chromophore with polar groups including the solvent. Therefore, we cannot rely on such generalities.

It should be realized that even in a somewaht simpler system, containing only one tryptophyl residue, we cannot assign unambiguously the specific molecular interactions that are responsible for the energy of the absorption band since it may result from several factors each of

which may be involved to different degrees. More generally several tryptophyl residues are present in the protein, some exposed and others buried in the protein interior. The protein absorption spectrum is a composite of the absorption spectrum of each aromatic residue and cannot be used directly as an indicator of the microenvironment that each chromophore enjoys. The absorption spectra of proteins, and a knowledge of solvent effects on tryptophyl absorption are used mainly in techniques such as U.V. difference spectroscopy and solvent perturbation studies to determine the degree of tryptophyl exposure to various solvents and in various states of protein conformation.

As we have seen, to a first order approximation, the absorption band of buried chromophores lies to the red of exposed tryptophyl residues. This agrees well with the fact that the incorporation of aromatic amino acids into proteins is usually accompanied by a red shift to longer wavelengths in their U.V. absorption spectrum compared to their absorption as free amino acids (35, 36). A comparable blue shift occurs upon protein denaturation (27). These types of shifts are readily observed as ultraviolet difference absorption spectra.

The chromophoric groups of most interest in these studies are tryptophan and tyrosine since phenylalanine makes a relatively small contribution to the total protein absorption spectra. We can establish some degree of resolution of protein absorption spectra into its tryptophyl and tyrosine components since their absorption occurs at slightly different energies. For example, the position of the maxima at 285, 278, 274, are characteristic of tyrosine and the maxima at 290, 283, and 273nm are characteristic of tryptophyl absorption in 20% ethylene glycol.

By observing the spectral shifts which occur in the absorption spectra of these aromatic amino acids upon partial or complete protein denaturation, U.V. difference spectra can give us a somewhat quantitative measure of the degree of exposure of tryptophyl and tyrosine residues in proteins. However, as we have seen, many factors are involved in producing the observed spectral shifts and changes in exposure alone may not explain the shifts completely. Several instances of spectral shifts that exhibit no corresponding changes in exposure are given by Kronman and Robbins in their review of this subject (37).

Part of the ambiguity that accompanies the spectral shifts observed by U.V. difference spectroscopy has been resolved by the use of solvent perturbation spectroscopy, first developed by Laskowski and Herskovits (38). This method is based on the fact that the position of a chromophores absorption band is dependent upon the solvent it is in. Thus depending on such solvent properties as dielectric constant, refractive index and hydrogen bonding ability of various solvents, one will observe red shifts (to longer wavelengths) or blue shifts (to shorter wavelengths) of the absorption spectra of these chromophores.

The general method involves difference absorption spectra of one solution of protein at fixed pH, ionic strength and concentration. The other solution is identical but has the perturbant added. The parameter monitored is the magnitude of the spectral shifts relative to a suitable standard. For example, the exposure of proteins to solvents of higher refractive index (more polarizable solvents) than water, results in a small change in the extinction coefficient and a small red shift of the absorption band. The magnitude of these changes depends on the location of the chromophores in the protein matrix. Those exposed to

the perturbing solvent would be expected to undergo the characteristic red shifts. Thus the degree of the spectral shifts of these chromophores absorption may be used to give an indication of the degree of exposure. Kronman and Robbins (37) provide a good review of the procedures involved in this technique, the limitations of the method and many examples of systems where solvent perturbation has been used to determine the exposure of tryptophyl and tyrosine residues (37).

One limitation of this technique is that perturbation with one perturbant alone cannot distinguish between a few completely exposed groups and a larger number of partially exposed groups. However, a series of perturbants varying in molecular weight and chemical properties can generally be used to discriminate between fully and partially exposed groups. In this manner aromatic residues located in crevices can be distinguished from residues on the molecular surface. The perturbants used are molecules such as sucrose, glycerol (long range perturbants) and methanol, polyethylene glycol and dimethyl sulfoxide (short range perturbants). Perturbants with short range effects are those that must complex or contact some part of the chromophore in order to accomplish the perturbation shifts of that chromophore's absorption spectrum. Long range perturbants are effective at greater distances since they are able to achieve the observed perturbation shift by nonspecific solvent effects. The use of different perturbants with different range effects may yield different degrees of exposure for partially buried groups.

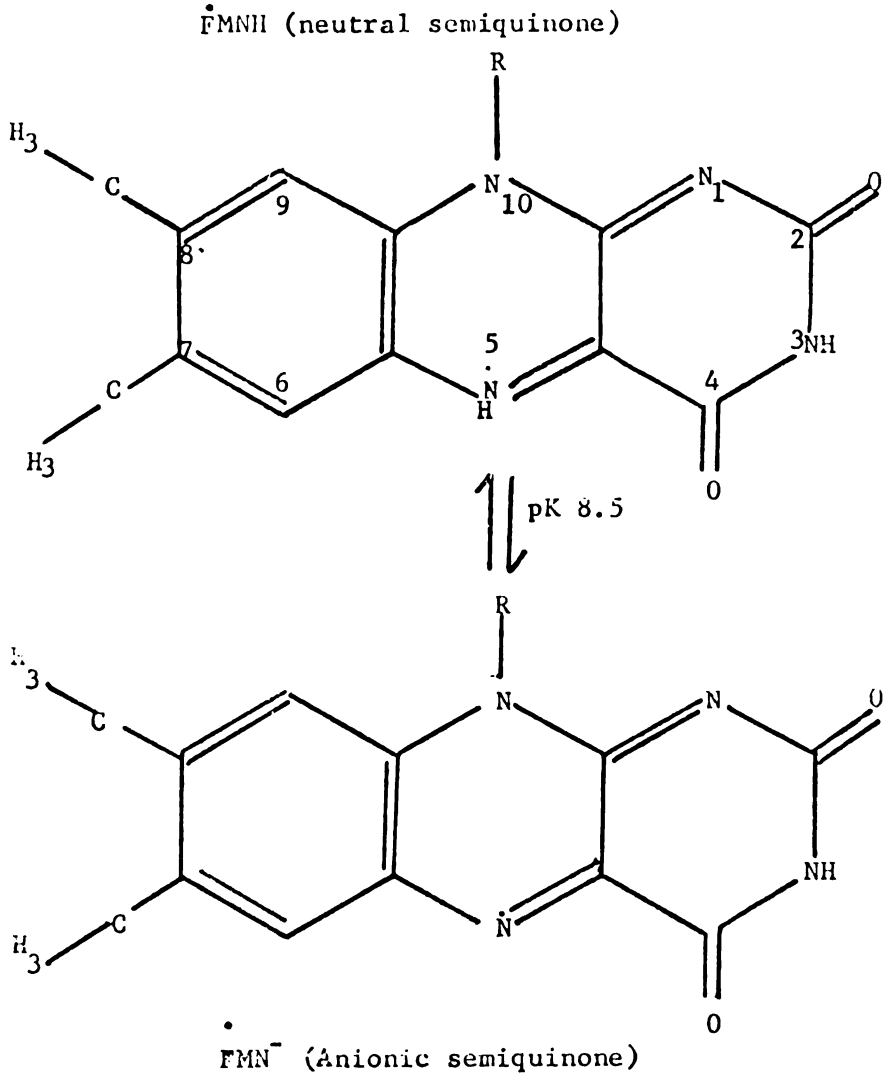
An undesirable aspect of solvent perturbation is that it may often disrupt the native protein structure. For example, short range perturbants will produce large spectral shifts, however, this is at the

expense of possibly disrupting the structure through the formation of new hydrogen bonds. When solvent perturbation techniques are coupled with other methods of tryptophan exposure analysis, such as chemical reactivity, the ambiguity between many partially exposed tryptophyl groups and a few fully exposed groups may be resolved (39).

Absorption spectroscopy may also be used to study the formation of charge transfer complexes between the exposed tryptophyl residues of proteins and specific electron acceptors. Characteristic of such complexes is a new absorption band which is observable in neither the donor nor the acceptor alone. Such a charge transfer complex, between n-methylnicotinamide chloride and the exposed tryptophyl residues of lysozyme has been studied by Deranleau et al. (40). The yellow complex formed between these two compounds was attributed to a charge transfer transition in which the indole ring was an electron donor and the n-methylnicotinamide chloride the electron acceptor. Using the degree of yellow color as a measure of complex formation they conducted titration studies and found that a weak complex (K_a, association constant =3.2 liters/mole) was formed with a single class of binding sites on the lysozyme molecule. Due to the extensive overlap of the protein absorption with the short wavelength absorption edge of the charge transfer absorption band, complete resolution of the charge transfer spectra was not possible and quantitative measurements of the number of complexing tryptophyl residues per protein molecule were not made. They suggest that the geometry for such complex formation requires that the ring faces of both the donor and acceptor be rather completely available for complexation.

A system in which an electron acceptor complexes specifically with

tryptophan, to yield a new absorption band fully separated from the absorption of the protein and the electron acceptor, would allow quantitative determination of the concentration of the complex and thus the number of tryptophyl residues of a protein which are "exposed" and able to complex with an appropriate electron acceptor. We have found that the charge transfer complex formed between flavin mononucleotide and tryptophan meets all of these requirements and provides a convenient and reliable method for determining the degree of tryptophyl exposure. The structure of tryptophan and FMN in its two physiological forms is shown in Figure V.



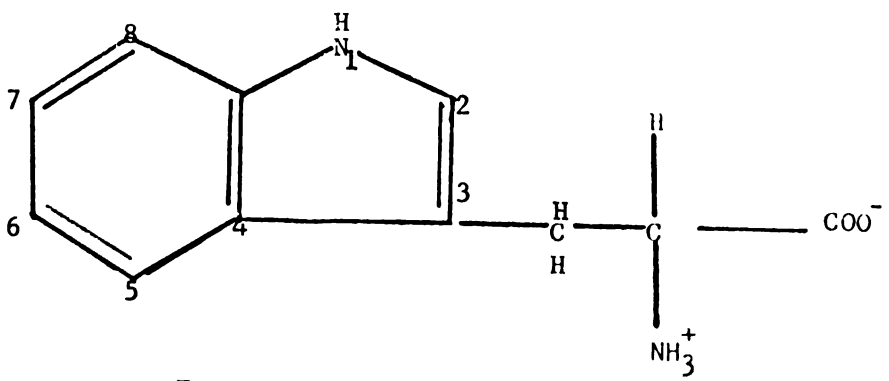


Figure V. Molecular Structure of FMN and Tryptophan.

Complex Formation Between FMN and Tryptophan

Isenberg and A. Szent-Gyorgyi (41) first noticed the red color that appears in solutions of Flavin mononucleotide (FMN) and tryptophan. When solutions of 10^{-2}M FMN and 10^{-2}M tryptophan are mixed together in solution, a red color is observed which is present in neither substance alone. As the temperature is decreased, an intensification of this color is observed. Realizing the excellent electron donating properties of the indole ring of tryptophan, the electron accepting properties of FMN and the similarity of the red color of FMN-tryptophan solutions to the red color of the acid reduced flavin semiquinone (FMN after accepting one electron) they suggested the formation of a 1:1 charge transfer complex:

FMN + tryptophan----- ($FMN_{reduced}$-$tryptophan_{oxidized}$) complex. In their argument, tryptophan is assumed to donate an electron to the FMN which is then reduced to its semiquinoid form. It is the flavin semiquinone which is responsible for the observed red color of the solution.

In Figure VI is the absorption spectra of FMN and a FMN-tryptophan solution in .1M phosphate buffer at pH 6.9. Several things should be noticed in these spectra. First, the absorption of the flavin is substantially reduced when tryptophan is present in solution. Since the extinction coefficient of oxidized flavin is higher than the extinction coefficient of reduced flavin, it has been suggested that the flavin absorption is decreased in flavin-tryptophan solutions as a result of reduction of the flavin.

Wilson (42) has shown that the oxidation-reduction potential of the flavin is reduced when it is in solution with tryptophan. This is explained as a decrease in the ratio of oxidized flavin to reduced

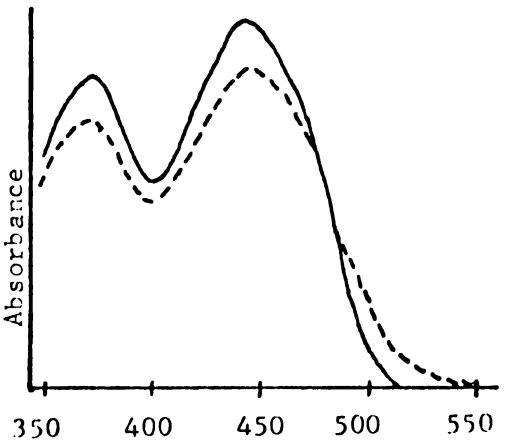


Figure VI. Absolute absorption spectra of FMN and FMN + TRP. (FMN alone in .1M phosphate buffer pH6.9 (———) FMN plus Tryptophan, $2x10^{-3}$M in same buffer (– – – –) concentration of FMN 10^{-5} M in each.

flavin (the O/R ratio). Both of these observations are compatible with an increase in the amount of reduced (semi-reduced) flavin in flavin-tryptophan solutions due to reduction of the flavin via accepting an electron from tryptophan.

Secondly the increase in absorption at the 450nm flavin absorption band in the presence of tryptophan should be recognized. This shoulder is clearly resolved in difference absorption spectra and appears with a maxima near 500nm.

The difference absorption spectra of 10^{-4}M FMN + $2x10^{-3}$M tryptophan balanced against 10^{-4}M FMN is displayed in Figure VII. A positive absorption maxima appears near 500nm and two negative peaks near 370 and 450nm. These negative peaks form a negative mirror image of the flavin absorption. They result from the presence of more oxidized flavin in the reference solution (which contains FMN alone) than in the sample solution (which contains tryptophan and FMN) where some of the oxidized flavin has been reduced. The positive difference absorption maximum near 500nm is characteristic of the absorption of the flavin semiquinone. This difference absorption band is not observed in solutions of FMN of tryptophan alone. Flavin in 10 per cent HCl is reduced to a red form by sodium hydrosulfide and also shows a difference absorption maximum of the semiquinoid form of flavin in 1M HCl, determined to be 503nm by Beinert(43). Successive stages in the reduction of FMN with zinc at acid pH shows the growth of a distinct absorption band with a maximum near 500nm. This is clearly shown in Figure VIII.

Slifkin (44) has shown that the broadening of the 450nm flavin absorption, as observed in FMN-tryptophyl solutions is not merely a perturbation of the flavin spectrum since both the spectra of FMN with

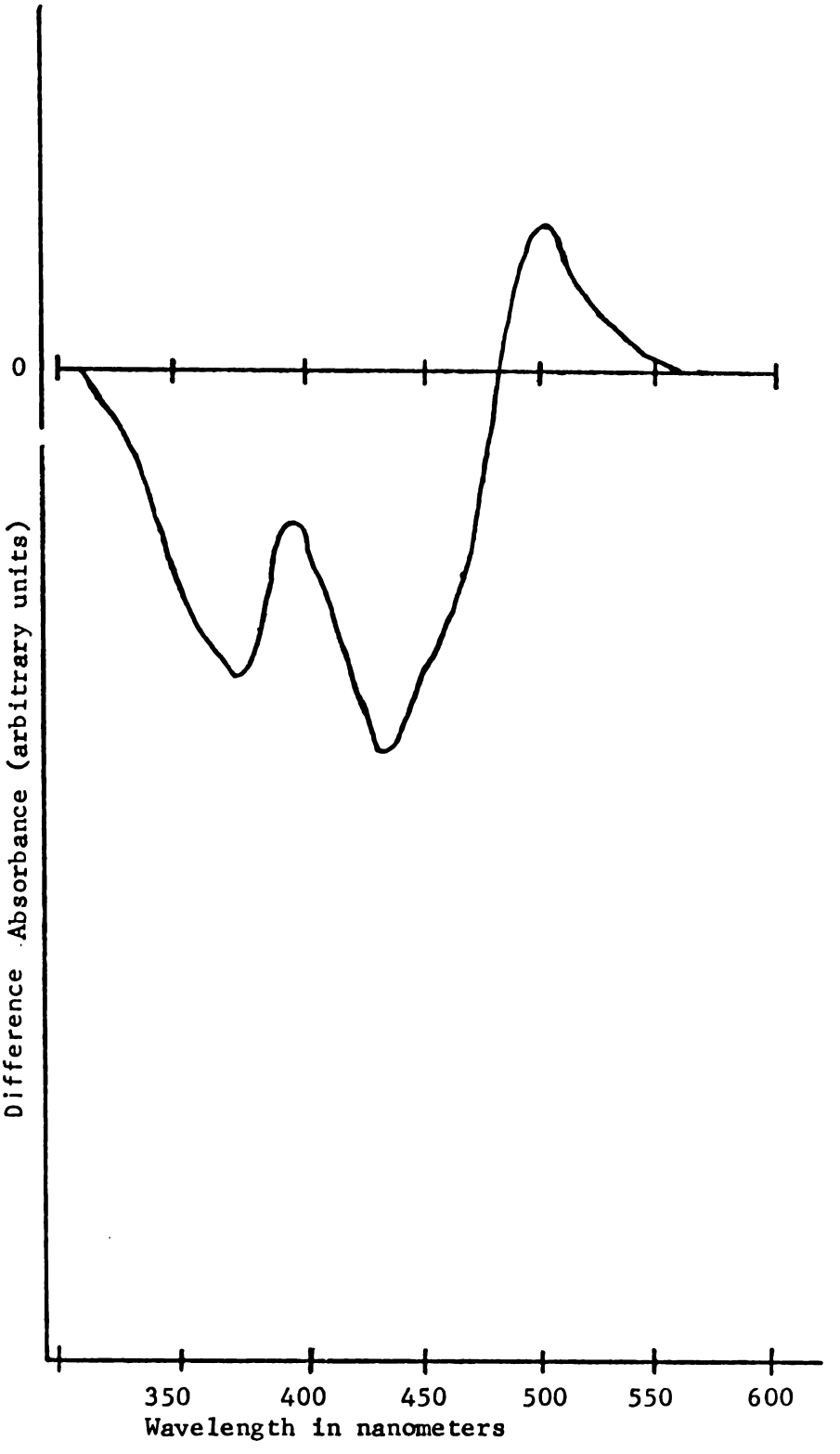


Figure VII. Difference absorption spectra of 10^{-4}M FMN and $2x10^{-3}$M Tryptophan. Sample cell contained FMN-Tryptophan, reference cell FMN alone.

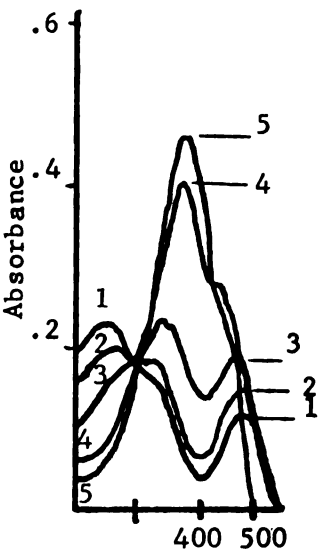


Figure VIII.
FMN in 1 N HCL in succesive states of reduction. curve 5 full oxidation, curve 1 about 75% reduction. curve 4 reoxidized material after previous reduction with Zn. Taken from figure 6 of reference (43).

increasing amounts of tryptophan and FMN in successive stages of reduction with zinc have not only difference absorption maxima near 500nm but also isobestic points near 330nm. This suggests that each spectra represents stages in the same process, namely the reduction of the flavin. Slifkin also points out that the absorption spectrum of some of the stronger complexes with FMN show quite clearly resolved bands near 500nm even in their absolute absorption spectrum (45).

The flavin free radical in solution alone is very unstable, with a decay constant of $4 \times 10^{8} M^{-1} sec^{-1}$ (46). In FMN-tryptophan complexes tryptophan donates an electron to the flavin reducing it to its semiquinone form which is stabilized at neutral pH by complex formation with tryptophan. Studies of the crystals of these complexes and polarographic measurements of FMN-tryptophan solutions have verified that these complexes enjoy a 1:1 stoichiometry (42, 47).

It is important to realize that the new difference absorption band at 500nm is the absorption band of the flavin semiquinone and not a charge transfer absorption band. The true charge transfer absorption band may be buried in the more intense absorption of the flavin. The production of the flavin semiquinone, with its characteristic absorption near 500nm, is evidence that charge transfer has occured. Since this absorption is not a charge transfer absorption but the absorption of a specific molecular species (the flavin semiquinone) its position is quite insensitive to the donor molecule used.

The lack of correlation between the ionization potential of various indole derivatives and the position of the difference absorption maxima in FMN solutions has been interpreted by some investigators as the lack of charge transfer complex formation between FMN and tryptophan. The

confusion here may have resulted from the false assumption that the 500nm difference absorption band is a charge transfer absorption band.

Isenberg (48) describes these complexes between FMN, indole, and indole derivatives as "strong charge transfer complexes" in which a great deal of charge is transferred from the electron donor (tryptophan) to the electron acceptor (FMN). The criteria which he uses to classify these complexes as strong complexes are as follows:

1. The complex is one between an electron donor and an electron acceptor.
2. The complex will have a new optical absorption band which is relatively insensitive to changes in the donor molecule and may appear quite similar to the absorption band of the free radical of the acceptor.
3. The complex can dissociate into radical ions in the absence of illumination, but with the proper environmental assistance.
4. The complexing has a "local" nature of some sort. In the case of indole or tryptophan, the donor electron is from a π orbital and the interaction occurs over a special small region of the molecule.

Isenberg has suggested that even after the flavin has accepted an electron, the donor and acceptor molecules may remain so close that the electrons are still coupled or held very near together. As evidence of this coupling after charge transfer is the fact that no ESR (electron spin resonance) signal is observed in associated FMN-tryptophan complexes in solution.

Theoretical electron density calculations of various amino acids by the Pullmans (49), suggest that tryptophan is the most effective electron donor of all the amino acids. This prediction has been supported by a number of independent studies: Isenberg and Szent-Gyorgyi (41),

Fujimora (50), Harbury et al. (51) and Cilento and Tedeschi (52).

Szent-Gyorgyi comments rather colorfully on the electron donating properties of the indole ring: "... if nature developed a substance for electron transfer, then she may have given to the substance extraordinary qualities as an electron transmitter." "Indole has such qualities which make it likely that nature developed this ring actually for services of this kind." "It is tempting to think that nature introduced this amino acid into the protein molecule to mediate its charge transfer reactions." (53)

Szent-Gyorgyi et al. (54) have suggested that the electron transfer from indole is localized on carbon atom 3 (C_3). Foster and Hanson studies of the chemical reaction between strong electron acceptors and indole support the conclusion that the C_3 of indole has the greatest electron density and is the localized site for electron donation (55). Green and Malrieu (56) have conducted quantum chemical studies of indole and indole derivatives. Their results indicate that indole is even a more efficient electron donor than expected because of the "localized" nature of its electron donation. Since FMN is a good electron acceptor (53) it is understandable that FMN forms strong charge-transfer complexes with tryptophan.

Further evidence that charge transfer forces play a role in stabilizing complex formation between FMN and tryptophan is presented by Wilson (42). He has shown that the oxidized neutral form of the isoalloxazine ring shows the highest affinity for indole and its derivatives. Only FMN, the better electron and not its semireduced or reduced forms (FMNH or $FMNH_2$) forms strong complexes with indole.

Such complexes should dissociate to a greater extent in solvents of

higher dielectric constant. This is observed for FMN-tryptophan complexes by their greater dissociation in the presence of ethanol. This effect has been observed by Pereira and Tollin (47), and Wilson. The dissociation is even greater in solutions of dioxane (42).

Although the complex between tryptophan and FMN could be stabilized by several types of forces in a geometry that allows the electron to pass over to the flavin to produce the flavin semiquinone there is evidence that charge transfer forces may serve to stabilize such complexes. Bowd et al. (57) have studied the quenching of FMN fluorescence by various indoles at different temperatures. Activation energies were obtained from the variation of the Stern-Volmer quenching constants at different temperatures. Not only were these energies on the same order as typical charge transfer complexes, but the addition of nucleophilic groups (which serve to decrease the electron-donating power of indoles) to the indoles increased the activation energy. The observation that strong complexes form in organic solvents suggest that electrostatic interactions are active in stabilizing these complexes (59). Harbury and Foley (58) have shown that hydrogen bonding plays no significant role in stabilizing such complexes. In addition, thermodynamic calculation on neutral flavin-indole complexes show that the entropy changes are always negative and thus hydrophobic forces are relatively unimportant in complex stabilization. Although forces other than charge-transfer may be involved in some extent in complex stabilization the production of the flavin semiquinone and its stabilization in FMN-tryptophan solutions does indicate that charge transfer has occured i.e. and electron has been transfered from the indole ring to the flavin. The above evidence suggests that charge-transfer forces are

involved in stabilization of the complex.

Two other essential criteria for charge transfer complexes is that any property associated with the presumed complex must be reversible with temperature and upon dilution. Both of these criteria hold for the FMN-tryptophan complex. Isenberg and Szent-Gyorgyi point out the intensification of the red color of such complexes as the temperature of the solution is lowered or as the concentration of either FMN or tryptophan is increased. The difference absorption maximum near 500nm is also observed to increase proportionally with reductions in temperature or increases in the concentration of either substituent.

Isenberg and Szent-Gyorgyi have observed this red complex formation with flavin to be specific for tryptophan among amino acids (41). They observed the presence of this red color only in solutions of FMN and tryptophan and not in solutions of FMN (10^{-3}M) and tyrosine, phenylalanine, nor histidine, at 10^{-3}M each. Decreasing the temperature enhances complex formation and vividly intensifies the red color of FMN-tryptophan solutions, yet even at -78 degrees C, solutions of FMN and other aromatic amino acids show no trace of redness. In agreement with their results we observed the absence of any difference absorption maxima near 500nm in solutions of 10^{-3}M FMN and concentrations of these aromatic amino acids of 3 x 10^{-3}M each and with 3 x 10^{-3}M insulin, a protein containing tyrosine and phenylalanine but no tryptophyl residues. However, a difference optical density of .22 is observed at these concentrations of flavin and 3 x 10^{-3}M tryptophan. These results suggest that at least at these concentrations (3 x 10^{-3}M) of amino acids, formation with FMN is specific for tryptophan.

Other authors have presented evidence for similar complex formation

between FMN and other amino acid derivatives. The phenols (60) and some aliphatic amino acids such as glycine, alanine, and leucine, are believed to complex with FMN (61). However all of these interactions are very much weaker than the complexes formed between FMN and tryptophan.

Fleishman and Tollin (60) have observed the formation of 1:1 complexes between electron donating phenol compounds and FMN. These complexes are more stable in neutral solutions than at acid pH similar to what has been reported for FMN-tryptophan solutions and are enhanced by decreasing temperature. The absorption spectra in acid solutions of one ring phenols such as orcinol, pyrogallol, and trimethyl hydroquinone are very similar to the absorption spectra of the semiquinone form of FMN and FMN-tryptophan solutions. No new absorption bands are present in the absolute spectra, only a large tailing of the flavin absorption to the red. With very electron rich double ring phenols such as 1,4-napthalenediol, distinct new absorption bands are observed near 500nm. Although these charge transfer complexes that form between one ring phenol derivatives are stronger than any of the complexes formed between FMN and any aliphatic amino acid they are still much weaker than FMN-tryptophan complexes. These results do indicate, however, that charge transfer complex formation is possible between FMN and the tyrosine residues of proteins even though such interactions are much weaker than with tryptophyl residues.

Similar complex formation is observed at higher concentrations of tyrosine and in flavinylpeptides of tyrosine where the flavin and tyrosine are intramolecularly linked so that complex formation is independent of concentration (59). Yet even in these flavinylpeptides

the complex formed in flavinyltryptophan is observed to be much stronger than that formed in flavinyltyrosine, Thus in the sense of its strong concentration dependence in flavin-amino acid or flavin-protein solutions such complex formation and the resulting contribution to the 500nm difference absorption, is highly specific for tryptophan.

Determination of K_a and $\Delta\varepsilon_m$ for the FMN-Tryptophan Complex

The association constant for the flavin-tryptophan complex and the molar extinction difference coefficient was determined by difference absorption spectroscopy. Complex formation results in the transfer of an electron to the flavin to produce the semireduced form which absorbs at 500nm. Thus the absorption at this wavelength is proportional to the amount of complex formation and the association constant of the complex. The increase in the 500nm difference absorption maxima was followed spectrophotometrically for 10^{-3}M flavin and solutions of tryptophan decreasing from $5x10^{-3}$M, in .1M phosphate buffer, pH 6.9. Under these conditions, the concentration of tryptophan greatly exceeds the concentration of the complex formed and a plot of reciprocal concentration of tryptophan versus the reciprocal of the absorbance at 500nm yields the straight line plot shown in Figure IX. This line intercepts the abscissa at $1/\Delta\varepsilon xr$ and the ordinate at $-K_a$ where K_a is the association constant of the complex. Here $\Delta\varepsilon$ is the molar extinction difference coefficient, r, the concentration of flavin, and x is the cell path length which was 1cm. The association constant, K_a, was determined to be 97 liters/mole and the molar extinction difference coefficient approximately 1100 liters/mole-cm.

This value of the association constant is in good agreement with the value of 92 liters/mole determined by Wilson (42) in the same solution

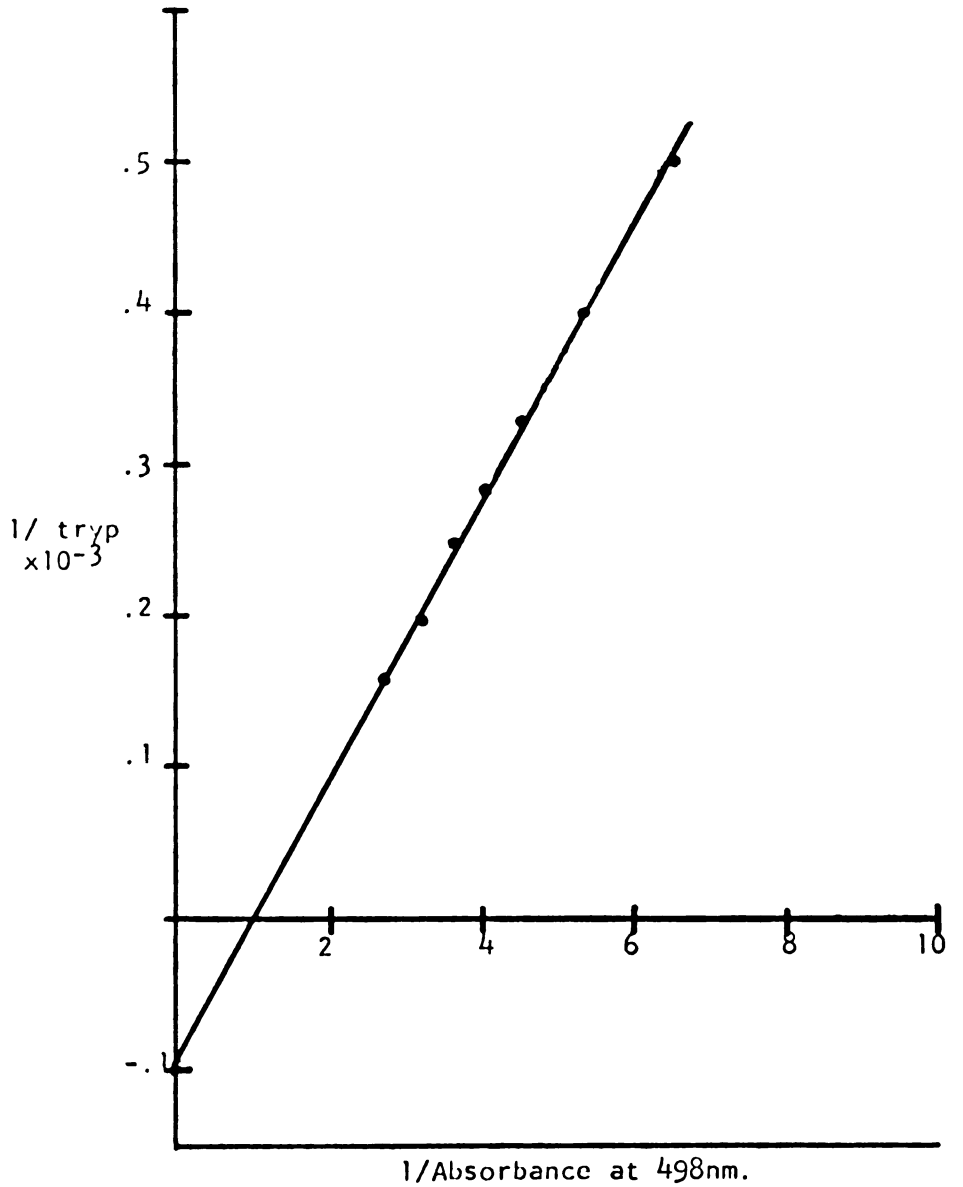


Figure IX. Determination of K_a and $\Delta\varepsilon M$

and the value of 98.4 liters/mole determined by Pereira and Tollin in .1m phosphate buffer with ethanol 3:7 (v:v) (47). Since the extinction coefficient for oxidized FMN at 500nm is approximately 2200 liters/mole-cm, the molar extinction coefficient of the complex is approximately 3300 liters/mole-cm, in good agreement with Wilson's value of 3580 liters/mole-cm.

Complex formation and Tryptophyl exposure

Because of the ease of monitoring complex formation spectrophotometrically and the specificity of complex formation for with tryptophan among amino acids, Swinehart and Hess first suggested that such complex formation might be a useful tool to determine tryptophyl exposure in proteins. They studied the binding of FMN to the exposed tryptophyl residues of alpha-chymotrypsin (62).

Under conditions where the number of exposed tryptophyl residues of chymotrypsin is sufficiently greater than the concentration of the FMN-tryptophan complex (i.e., chymotrypsin at 1.8-10 X 10^{-4}M and FMN at 1.1 X 10^{-4}M) a plot of reciprocal concentration of chymotrypsin versus reciprocal absorbance near the 500nm difference absorption maxima yielded a straight line which intercepts the ordinate at nK. Here n is the number of tryptophyl residues which are available for complex formation with FMN in solution and K is the association constant of the complex. Similarly under conditions where the concentration of FMN is sufficiently greater than the concentration of complex (i.e. 1.8-6.7 X 10^{-4}M FMN and 3 X 10^{-4}M chymotrypsin) a plot of reciprocal flavin concentration versus reciprocal absorbance at the difference absorbance maxima gave a linear plot which intercepts the ordinate at K. Division of nK by K was used to determine n, the number of exposed tryptophyl residues

of chymotrypsin available for complex formation with FMN in solution. In this manner they determined n=0.8 for chymotrypsin in .05M acetate buffer at pH 5.0. In the same buffer and concentrations of chymotrypsin from $1.8\text{-}10\text{X}10^{-4}$M and FMN at $1.1\text{X}10^{-4}$M they determined nK to be 350 liters/mole and $\Delta\epsilon_m$ to be 1800 liters/mole-cm.

Besides the difficulty involved in the number of spectral measurements, the large concentrations of protein that such analysis require may be inhibitive for the general use of this technique. Another difficulty with this approach results from the fact that chymotrypsin undergoes a pH, ionic strength, and concentration dependent dimerization (63, 64) and changes in tryptophyl exposure have been reported upon dimerization (65). Thus such results are confused by the concentration dependent changes in monomer-dimer equilibrium which in themself may result in changes in tryptophyl exposure. Our results, which are presented in detail later, show from 2 to 3 tryptophyl residues exposed and available for complex formation with FMN. These results are in good agreement with the values of tryptophyl exposure determined by X-ray analysis, solvent perturbation, and chemical reactivity studies of tryptophyl exposure in this protein. Using n=2 and n=3 and nK=350 liters/mole from Swinehart and Hess's data, K is determined as 116-175 liters/mole and $\Delta\epsilon_m$ 1700-1800 liters/mole-cm. Such values are in reasonable agreement with those we obtained in our work and the work of other investigators as mentioned earlier. Some variation in these values may arise also from the differences in ionic strength, pH, and buffers used in each case.

We have found that the charge transfer complex formed between FMN and the "exposed" tryptophyl residues of several proteins can be easily

monitored by following, spectrophotometrically the 500nm difference absorption band which is the characteristic absorption of the flavin semi-quinone and thus indictive of complex formation. The use of standard curves of complex formation provides a convenient and reliable means of determining tryptophyl exposure in proteins, both in their native and denatured states.

In solutions of FMN, as the concentration of tryptophan is increased, we would expect more complex formation and thus a greater optical density at the 500nm peak. Indeed, as seen in the standard curve, Figure X, there exists a nearly linear relationship between increasing optical density of the complex and increasing concentrations of tryptophan for $1x10^{-3}$M FMN and concentrations of tryptophan ranging from $2x10^{-4}$M to $4x10^{-3}$M in .1M phosphate buffer, pH 6.9. It should be recalled here that the new 500nm difference absorption peak arises from the absorption of the flavin semi-quinone which is produced and stabilized by complex formation with tryptophan. Although this is the absorption of the flavin semiquinone, we refer to the 500nm difference absorption as the "complex optical density" since complex formation is the necessary prerequisite for the production of the flavin semiquinone.

After preparing known concentrations of the proteins in solutions of 10^{-3}M FMN in .1M phosphate buffer, pH 6.9, we determine the optical density of the difference absorption peak near 500nm by difference absorption spectroscopy. From the optical density of this complex, we determine (from the standard curve of FMN-tryptophan complex optical density-vs-concentration of tryptophan) the concentration of free tryptophan in solution with 10^{-3}M FMN, whose optical density corresponds to the optical density of the protein-FMN complex. This gives us the concen-

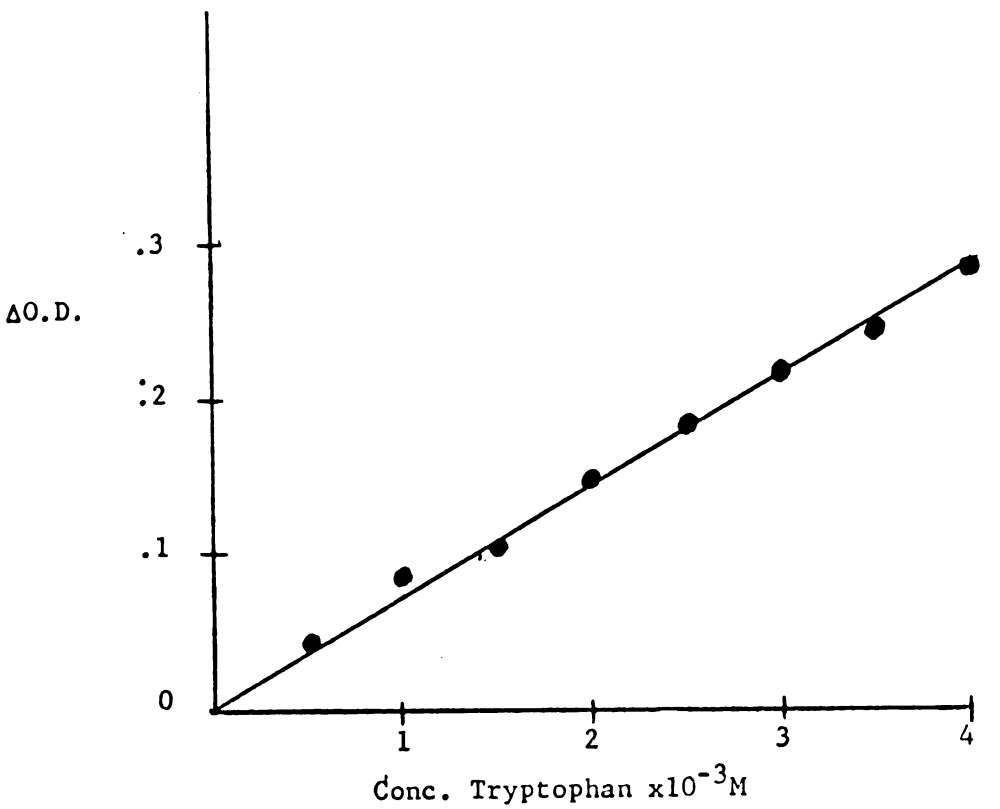


Figure X. Standard Curve.
ΔO.D. (10^{-3}M FMN + Tryptophan-vs- 10^{-3}M FMN) -vs- Conc. Tryp.
.1M Phosphate buffer, pH 6.9, 23°C.

tration of the tryptophyl residues of the protein that are available for complex formation with FMN. Dividing the concentration of the tryptophyl residues of the protein that are available for complexing with FMN by the actual concentration of the protein used, we determined the number of "exposed" tryptophyl residues per protein molecule, N.

One assumption that is implicit in this study is that the incorporation of the tryptophyl residue into the polypeptide chain does not in itself alter the affinity of tryptophan for complex formation with FMN. To test the validity of this assumption three proteins (lysozyme, alpha-chymotrypsin, and trypsin) were heat denatured in the presence of FMN and their difference absorption spectra recorded at 23°C. From the optical density of the difference absorption peak near 500nm the concentration of tryptophyl residues available for complex formation with FMN per protein molecule was calculated. In each case the number of tryptophyl residues complexing was equal to the total number of tryptophyl residues of the protein (See table II). This shows that the affinity for FMN is not altered by its incorporation into the polypeptide chain.

The degree of complex formation with tryptophyl residues would be altered, however, if a residue is buried in the interior of the protein or a crevice in the protein structure such that complex formation with FMN was impossible. Thus the values that we obtained for tryptophyl exposure are given as N= the number of tryptophyl residues per protein molecule that are available for complex formation with FMN in solution. These N values are presented in Table II with the literature values of tryptophyl exposure in these proteins.

Materials and Equipment

All absorption spectra were taken on a Cary model 15 recording

Table II. **Experimental results and Literature Values of Tryptophyl Exposure.**

Enzyme	State	Concentration $x(10^{-4}M)$	trials	Difference Absorbance $\pm$ s	N$\pm$ s (exposure)
Alpha-Chymotrypsin					
	native	2.0	5	.030 $\pm$.004	2.04 $\pm$ .27
		2.5	5	.040$\pm$ .002	2.18 $\pm$ .11
		3.0	5	.051 $\pm$ .004	2.31 $\pm$ .18
	denatured	2.0	3	.11 $\pm$ .005	7.5 $\pm$ .34
		2.5	2	.15 $\pm$ .004	8.2 $\pm$ .22
		3.0	1	.17	7.7
Lysozyme					
	native	2.0	5	.060 $\pm$ .004	4.08 $\pm$ .27
		2.5	5	.077 $\pm$ .004	4.18 $\pm$ .21
		3.0	5	.091 $\pm$ .005	4.12 $\pm$ .23
	denatured	2.5	2	.106 $\pm$ .006	5.8 $\pm$ .33
		3.0	2	.135 $\pm$ .005	6.1 $\pm$ .23
Trypsin					
	native	3.0	3	.048 $\pm$ .004	2.2 $\pm$ .18
		4.0	1	.060	2.0
	denatured	3.0	2	.093 $\pm$ .005	4.2 $\pm$ .23
Pyruvate Kinase					
	native	1.0	3	.057 $\pm$ .003	7.8 $\pm$ .41
Insulin					
	native	3.0	2	0.0	0.0

Literature values:

Enzyme	Total TRYP	X-ray	Solvent Perturbation	Chem. Reactivity
Lysozyme	6	4	4, 3-4, 4-5	4
Chymotrypsin	8	2	2 ,2-3	2-3
Trypsin	4		2	1-2
Pyruvate Kinase				5
Insulin	0			

spectrophotometer using 1 cm square quartz cells. The base line for each difference absorption spectra was obtained from 10^{-3}M FMN in both the sample and reference compartments. The sample cell was then filled with a known concentration of protein in a 10^{-3}M FMN solution. The absorption was zeroed at 610nm where no absorption of FMN, protein nor complex occurs. Difference absorption spectra were then recorded to 450nm. The optical density of the 495-500nm absorption peak was taken as the optical density of the charge transfer complex for that particular protein-FMN solution.

Each solution was prepared with a .1M phosphate buffer ph 6.9, except for the pyruvate kinase solution which was prepared from a $2x10^{-2}$M imidazole (10^{-3}M EDTA) buffer at pH 7.3. Determinations of pH were made with a Corning pH meter, model 12, and calibrated against standard buffers of pH 4.01 and 7.0.

Riboflavin-5'-phosphate (FMN) was obtained commercially from Calbiochem (B grade) and L-tryptophan from Eastman Organic Chemical Co. N.Y. and Calbiochem. Alpha-chymotrypsin (63u/mg), 3X crystalized and salt free, lysozyme (10,906 u/mg) and salt free, trypsin 2X crystalized and salt free and insulin 2X crystalized and salt free were all obtained from Worthington Biochemical Corporation Freehold, N.J. and were used without further purification. Pyruvate kinase was purified by a modified method of Tietz and Ochoa (66) as described by Kayne and Suelter (67). The enzyme was assayed by a coupled reaction of ADP, PEP (phosphoenol pyruvate) and NADH in the presence of 10^{-2}M MgCl and .1M KCl. The specific activity was determined to be between 150 and 250 micromoles of product/min-mg. of protein at 23°C.

Discussion of results

Our results agree quite well with estimations of the degree of tryptophyl exposure in lysozyme, trypsin, alpha chymotrypsin, pyruvate kinase and insulin as determined by other methods of exposure analysis.

Lysozyme

Solvent perturbation and chemical oxidation studies of lysozyme have not shown tremendous agreement and perhaps the most reliable results are found in those studies that agree with the evidence presented by X-ray crystallographic techniques. X-ray diffraction studies of lysozyme indicate that of the six total tryptophan residues (residues 62, 63, 108, 123, 152, and 153) four tryptophyl residues (62, 63, 108, and 123) lie on the molecular surface (68, 69). Residues 62, 63, and 108 are believed to lie within the active site cleft of this enzyme (68). Solvent perturbation studies with short range perturbants (methanol, dimethyl sulfoxide, and polyethylene glycol) indicate that from three to four tryptophyl residues are exposed. Perturbation with long range perturbants such as ethylene glycol, glycerol and sucrose, which should penetrate more deeply into the active site, seem to be more reactive with residue 108, while they still react with 62, 63, and 123. These long range perturbants show from four to five groups reacting (70). It thus seems likely that residues 62, 63, 108, and 123 are "exposed" while residues 28, and 111 are apparently "buried." Our results indicate four residues are exposed to FMN complex formation in solution in the native enzyme and six residues in the denatured enzyme, in good agreement with these other results.

Trypsin

In trypsin two tryptophyl residues were observed to be relatively

reactive towards hydrogen peroxide + 10 per cent dioxane, while a third residue was somewhat less reactive (71).

At pH 5.5 two tryptophyl residues were found to react with NBS while only one residue was found to be reactive at pH 7.0 (72, 73). Solvent perturbation studies reveal two partly or fully exposed tryptophyl residues (75). In the denatured enzyme 4.2 residues have been found to be reactive with HNBB (74). In 8M urea denatured enzyme, four residues have been found to react with NBS (73). These studies of reactivity with the denatured protein are in good agreement with amino acid analysis which indicates that trypsin has four total tryptophyl residues.

All of these results are in reasonable agreement with our finding that two tryptophyl residues of the native enzyme and four tryptophyl residues of the denatured enzyme are available to FMN for complex formation.

Alpha Chymotrypsin

N-Bromosuccinimide (NBS) chemical oxidation studies and solvent perturbation techniques indicate that there are three exposed tryptophyl residues in chymotrypsinogen, the zymogen of chymotrypsin (70, 76, 77, 80). Activation of the zymogen to chymotrypsin apparently produces some "tightening" of the protein structure about its tryptophan residues. NBS and H_2O_2 oxidation studies at pH 5.0 indicate that one of the three fully exposed tryptophyl residues of chymotrypsinogen becomes partially buried after activation of the enzyme, leaving two fully and one partially exposed tryptophyl residues (76, 77). In another chemical reactivity study one to three and three tryptophyl residues of alpah chymotrypsin were found to react with HNBB (2-Hydroxy-5-nitrobenzyl bromide) in the native state while 8.1 residues were found to react in 8M urea

denatured solutions of this enzyme (78, 79). Solvent perturbation studies also indicate that of the three fully exposed residues in the zymogen, one becomes partially buried after its activation to chymotrypsin (70, 80, 81).

An extensive report of the Three-Dimensional structure of crystaline alpha-chymotrypsin, at two Angstroms resolution by Birktoft and Blow (82) allows examination of the state of the tryptophyl residues of this enzyme. Of the eight total tryptophyl residues, six residues (tryp 51, 141, 172, 207, 215, and 237) are classified as surface residues by the positioning of their aromatic rings near the molecular surface. To examine the state of these residues more closely we have studied a three-dimensional model of the alpha-chymotrypsin molecule constructed from electron density contour maps through X-ray diffraction by A. Tulinski (83). This model shows two tryptophyl residues in the enzyme which should be readily available for charge transfer complex formation with FMN. These two residues (215 and 51) lie on the surface of the protein. The indole ring of their side chains are enough removed from other structural moieties of the protein such that no great hinderance of FMN binding to these residues should occur. Tryptophyl residue 207 which also lies on the molecular surface is somewhat more hindered from complex formation with FMN than residues 215 and 51 but a much more favorable candidate for complex formation than the remaining surface residues (141, 172, and 237). We cannot definitely say whether or not complex formation occurs with this residue.

Our results which show two or slightly more than two tryptophyl residues as being exposed to FMN binding in the native enzyme and eight residues exposed in the heat denatured enzyme are in good agree-

ment with X-ray diffraction analysis, solvent perturbation and chemical reactivity studies of tryptophyl exposure in alpha-chymotrypsin.

Pyruvate Kinase (rabbit muscle)

Very little conclusive data are available on the degree of tryptophyl exposure of this enzyme. Kayne and Suelter (84) have reported 4.6 ± 0.8 tryptophyl residues reacting with HNBB in the nonactivated enzyme using the method of Horton and Koshland (7). Titration with NBS by the method of Patchornik et al. (85) and the spectrophotometric method of Bencze and Schmid (86) suggest that there are twelve to fourteen tryptophyl residues per molecule of enzyme (87). Our results indicate a somewhat higher degree of exposure, eight residues are found to complex with FMN for the nonactivated enzyme.

Insulin

The absence of any 500nm absorption in the difference spectra of FMN + $3x10^{-4}$M insulin -vs- FMN suggest that there are no exposed tryptophyl residues in this enzyme. This is compatable with the fact that insulin contains no tryptophyl residues. These results also indicate that the two histidine residues of this enzyme, that are "exposed," (88) and the three exposed tyrosyl residues of insulin (89) do not complex with FMN. Further supporting the specificity of FMN complex formation with the "exposed" tryptophyl residues of proteins.

Comments on this technique of Tryptophan Analysis

The discrepancies between the findings of many solvent perturbation and oxidative reagent studies with one another and with the findings of X-ray analysis, point out the need of less perturbing and more sensitive and reliable probes for tryptophan analysis. Chemical methods which are often not specific for tryptophyl residues and which may in themselves alter the degree of tryptophyl exposure cannot give correct evaluations of the degree of tryptophyl exposure in the native enzyme.

We have found that many of the properties of the complex formed between FMN and tryptophan or the "exposed" tryptophyl residues of proteins, make FMN an ideal probe for tryptophyl exposure. Swinehart and Hess (62) point out that the presence of FMN in solution and the complex formation between FMN and the tryptophyl residues of alpha-chymotrypsin, does not in itself effect the degree of tryptophyl exposure, since their values of tryptophyl exposure were the same with FMN in excess of chymotrypsin and with chymotrypsin in excess of FMN. Such complex formation would certainly be expected to be far less perturbing to the native state of the enzyme than such harsh reagents as NBS, HNBB, and hydrogen peroxide which often result in the cleavage of the peptide bond.

A good reagent for exposure analysis should be specific for a certain amino acid. Formation of the complex between FMN and tryptophan, as observed by its characteristic 500nm difference absorption maxima, is specific for tryptophan among amino acids and for proteins which contain exposed tryptophyl residues. Even in the presence of high concentrations of other amino acids (3×10^{-3} M) no contribution to the 500nm difference absorption maxima is observed.

This complex formation is easily monitored by following the

intensity of this difference absorption maxima. This long wavelength absorption which results from complex formation and the production of the flavin semiquinone is enough removed from the absorption of the protein and the absorption of FMN to easily be followed by difference absorption spectroscopy.

A good reagent for exposure determination should be soluble in aqueous solutions, relatively stable over a relatively wide pH range and temperature range, and the reaction rate should be relatively fast. FMN is easily dissolved in aqueous solutions and as mentioned previously the reaction has been reported to be practically instantaneous and the complex stable even at $60^{o}C$ (90). The complex is somewhat sensitive to changes in pH. At very acidic pH, complex dissociation is observed (91). In the pH range 3.0 to 8.0 and in solutions of ionic strengths from 0.01 to 1.0 at pH 6.9 the complex is stable (42).

When we wish to study tryptophyl exposure in a protein under different environmental conditions, we can do this easily by preparing standard curves (as in figure X) under conditions that are identical to those where we wish to study the protein (i.e. the same pH, ionic strength, temperature, buffer etc.). In this way we negate any environmental influences that might alter the association of the complex and can thus determine accurately the degree of tryptophan exposure under various conditions.

A sufficiently high concentration of protein is necessary to insure that the optical density of the complex is large enough to be observed and recorded in a reproducible manner. Since concentration of tryptophan greater than $4x10^{-4}$ M are easily observed and reproducible, this is only a serious drawback when studying very scarce enzymes.

One assumption inherent in this technique is that the association of FMN with free tryptophan is the same as its association with the exposed tryptophyl residues of proteins. Kronman and Robbins (4) point out that if a side chain of an amino acid is located on the surface of a protein molecule so that a potentially active group projects into the medium and does not interact strongly with other structural moieties of the protein, then one would expect that this group should react with a suitable reagent at a rate comparable to that of the free amino acid having the same functional group. In this case the reactive group is the indole ring of the tryptophyl residue, and the reagent is FMN, and the reaction is complex formation and the transfer of an electron from the indole ring of tryptophan to the flavin. Our observation that the degree of such complex formation in the denatured protein is identical to the degree of complex formation with concentrations of free tryptophan that are equivalent to the total concentrations of free tryptophan residues in that protein suggest that merely being in the polypeptide chain does not alter the reactivity of a tryptophyl residue with FMN.

The reactivity of tryptophyl residues with FMN would be different from the reactivity of free tryptophan molecules in solution with FMN, if the side chain of the tryptophyl residue is hindered from complex formation, by interaction with some structural moiety of the protein. An extreme case of such lowered reactivity is of course when a tryptophyl residue is "buried" in the interior of the protein. In this case the side chain of the residue is clearly not available for complex formation with free flavin in solution. Other less extreme cases are possible of course, and any interaction of a tryptophyl residue with the protein which blocks complex formation would be responsible for our

assignment of this residue as "buried," with respect to its availability for complex formation with flavin. Thus we describe tryptophyl exposure in terms of the availability of tryptophyl residues of the protein for complex formation with FMN in solution. The general agreement of our "exposure" calculations with the results of chemical reactivity, solvent perturbation and X-ray studies suggest that a similar criterion for the assignment of "buried" and "exposed" groups in all of these methods. This is presumably the necessity of the exposure of the indole ring of tryptophan to the solvent and reagent such that the reaction occurs.

The Flavodoxins

The flavin-linked dehydrogenases carry either FMN or FAD (flavin-adenine dinucleotide) as prosthetic groups. They are important in catalyzing electron transfer to and from other proteins. The flavodoxins represent a subclass of the flavin-linked dehydrogenases. Flavodoxins are small flavoproteins dehydrogenases of molecular weight between 15,000 and 20,000. They serve as low potential electron carriers and each contains one FMN moiety as its only prosthetic group. These proteins act as substrates themselves for proteins and do not react directly with oxidizable substrates. They serve in many oxidation-reduction reactions transfering electrons to and from other proteins. In this process the FMN moiety is usually reduced while the "substrate" is oxidized. This reduction is generally shown as the transfer of hydrogen atoms from the "substrate" to the N_5 and N_1 positions of the flavin. This process is now known to consist of two steps, one yielding a stable semiquinone form (partially reduced flavin) and the second step yields the fully reduced flavin. Although the exact mechanism of this electron transfer is unknown, it is clear that the flavin group itself acts as an electron collection point from different "substrates."

The semiquinone forms of various flavodoxins were produced artificially by reduction with $Na_2S_2O_4^-$, by a photoinduced reduction with EDTA or by reduction with the substrate. Two different and distinctive absorption spectra were observed with various flavoproteins (92).

The absorption spectra of those flavoproteins which function as oxidases and hydroxylases was characterized, by a red semiquinone. This red radical has an absorption peak with a high extinction coefficient in the region of 370nm and another absorption maxima near 490nm.

Correlated absorption and ESR studies with model flavins have shown that this red radical species corresponds to the anionic flavin semiquinone. That is, FMN^{-}, the flavin with the accepted electron localized at the N_5 position and the N_1 and N_5 positions unprotonated. While the flavin semiquinone is unstable as a free radical in solution it is somenow stabilized by the protein in this red anionic form (93). The absorption spectra of flavoproteins which function as dehydrogenases are characterized by their purple-blue semiquinone form. Their semiquinones show an absorption band with a maxima in the 570-600nm region with a high extinction coefficient. Similar correlative absorption, ESR studies with model flavins have shown this form to be the neutral flavin semiquinone, FMNH, the flavin with the accepted electron at the N_5 position with the N_5 position protonated and the N_1 position still unprotonated (94). This free radical is also unstable when in solution but it is also stabilized by some protein-flavin interaction as the blue flavin semiquinone.

The conversion from red to blue semiquinone involves only protonation at N_5. This is shown in Figure IV with a pK of approximately 8.6 (94). Thus characteristic of flavoproteins is their ability to stabilize either red anionic semiquinone (oxidases) or the blue neutral semiquinone (dehydrogenases).

Edomondson and Tollin (95) have found that the oxidases and dehydrogenases have distinctly different circular dichroic spectra indicating that the flavin-protein interactions are of a somewhat different nature in each of these classes of flavoproteins.

With the dehydrogenase family, analysis of the band positions and rotational strengths of the vibronic transitions in the visible circular dichroic spectra of the oxidized, semiquinone, and hydroquinone (fully-

reduced) forms suggest that the nature of the flavin-protein interactions are quite similar in each form (95).

A detailed analysis of U.V. and visible circular dichroic spectra, protein fluorescence, fluorescence excitation spectra of these proteins, and differences in FMN and riboflavin binding ability support the further division of the flavoprotein dehydrogenases into two broad subclasses (95). One class is exemplified by the flavodoxin Clostridial Pasteurianum (Cl. Past.), its members include CL. Past., Clostridial M.P. (Cl.M.P.) and Peptostreptococcus elsdenii (P. elsdenic) and are referred to as pasteurianum type. The second class is exemplified by Rhodospirillum rubrum (R. rubrum) and includes R. rubrum and Desulfovibrio vulgaris (D. vulgaris). The Shethna Azotobacter vinelandii flavodoxin seems to be more similar to this latter class of flavodoxins, the rubrum type. The differences that define these classes of flavodoxins presumably result from differences in flavin-protein interactions in each class. D'anna and Tollin have suggested that the differences between the CD spectra and flavin binding characteristics probably result to some extent from the degree and type of conformational change which occurs upon flavin binding to the apoprotein.

In addition to the formation of the blue neutral semiquinone the family of flavodoxin dehydrogenases have another very interesting characteristic feature. Upon binding of the FMN moiety to the apoprotein the fluorescence of both the flavin and the protein is nearly completely quenched. Both flavin and protein fluorescence quenching obey the same second order rate law with identical rate constants for both flavin and protein fluorescence quenching (95). While the quenching of protein fluorescence is approximately 90% efficient in Cl. Past.

P. elsdenii, D. vulgaris, Cl. M.P. and Shethna Azotobacter flavodoxin, it is only 40% efficient in R. rubrum flavodoxin (96, 97, 98, 99).

Upon FMN binding to the flavodoxins several interesting changes occur: The extinction coefficients of the visible flavin absorption bands are dimished, the 450nm flavin absorption band is broadened and a red shoulder appears. The position of the red shoulder varies being near 475nm for Azotobacter and Clostridial flavodoxins and near 486nm for D. vulgaris (95, 100). These shoulders are very similar to the shoulders in the 450nm absorption band that arise upon partial reduction of free flavin and yet they occur upon FMN binding to the flavoprotein. The oxidation-reduction potential of the flavin is also substantially reduced upon flavin binding (101, 102, 103). It is interesting to note here that the occurance of a red shoulder or the broadening of the flavin absorption in the 450nm region, the decrease in the extinction coefficients of the flavin absorption and the decrease in the oxidation-reduction potential that occur in these flavodoxins are very similar to the changes that occur in solutions of flavin and tryptophan where complex formation occurs. These spectral similarities are pointed out even more clearly in Figure XI which is a comparison of the difference absorption spectra of the Shethna Azotobacter apoprotein-bound flavin-vs-unbound flavin in .025M phosphate buffer at pH 7.0, (dotted spectra) and the difference absorption spectra of 10^{-4}M FMN+2x10^{-3}M tryptophan-vs-10^{-4}M FMN in .1M phosphate buffer pH 6.9. The former spectra is taken from Edmondson and Tollin Figure 5 of reference (95). It is important to note the similarity of these spectra.

The Flavodoxin-flavin complex difference spectra shows one positive peak near 488nm with a difference molar extinction coefficient of

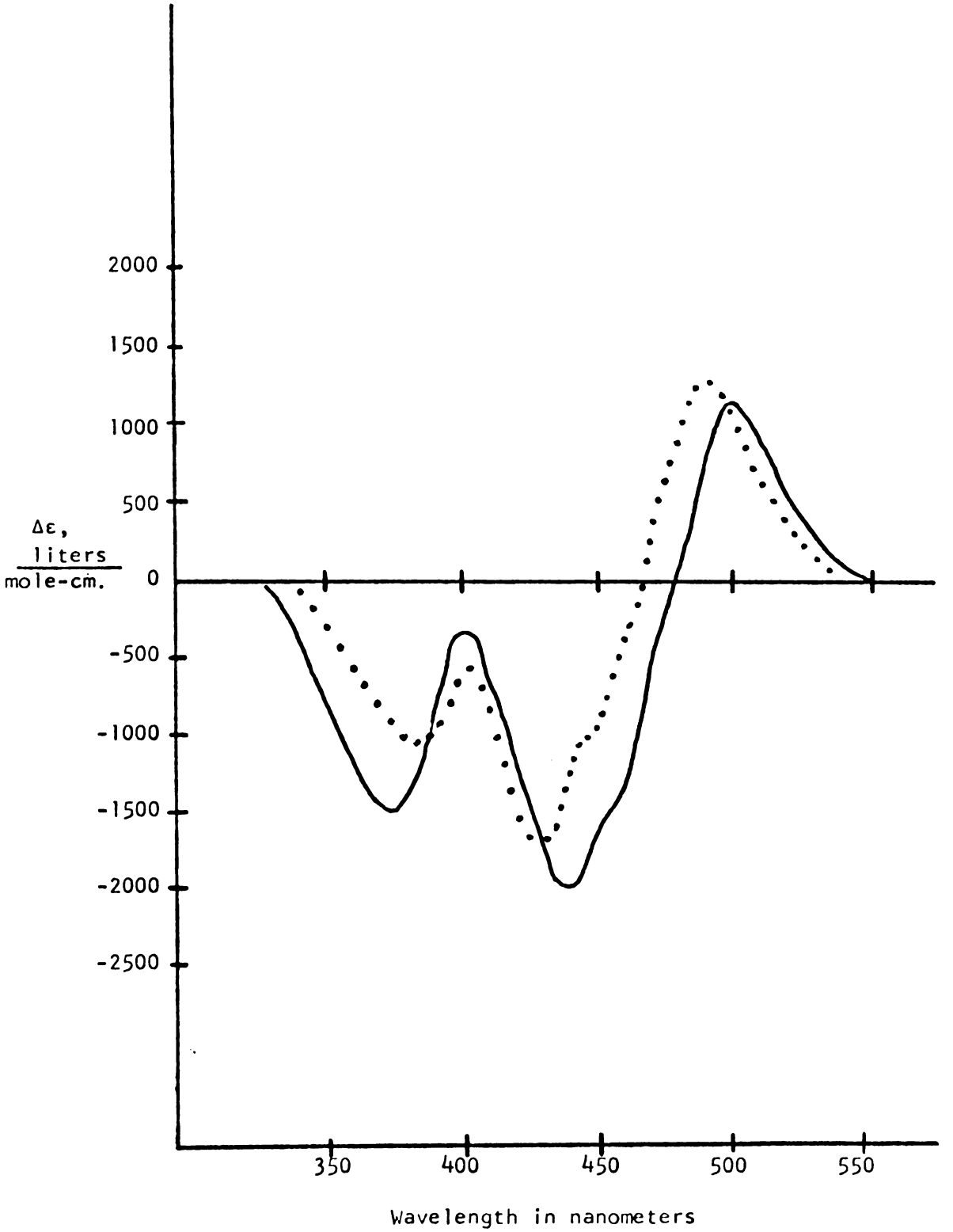


Figure XI. Difference absorption spectra of FMN-Tryptophan-vs-FMN (——) and Shethna azotobacter flavodoxin-bound FMN-vs-free FMN (....). The latter spectrum taken from Figure 5 of reference 95.

approximately 1300 liters/mole-cm. and two negative peaks near 433nm and 389nm. The difference absorption spectra of the FMN-tryptophan solution is very similar in that it shows one positive peak near 498nm with a difference extinction coefficient of approximately 1100 liters/mole-cm. and also two negative peaks at 439 and 379nm. Also difference absorption spectra of FMN and chymotrypsin is very similar to each of these difference spectra exhibiting one positive peak near 498nm with an extinction coefficient of approximately 1700 liters/mole-cm. and two negative peaks near 437 and 373nm. Müller et al (104) have reported similar difference absorption spectra for the Clostridial flavodoxins. The difference absorption spectra of these flavodoxins with bound flavin-vs-free FMN show one positive peak slightly above 500nm and two negative peaks near 440 and 370nm.

The difference spectra displayed in Figure XI of FMN-tryptophan solutions or of FMN and the exposed tryptophyl residues of alpha-chymotrypsin is the characteristic difference absorption spectra of the flavin semiquinone stabilized by complex formation with tryptophan, in such solutions. Because of its similarity to the difference absorption spectra of flavodoxin-bound flavin-vs-free flavin, we could believe that a similar FMN-tryptophan complex was formed between FMN and a tryptophyl residue at the FMN binding site of the flavodoxin if we knew there were a tryptophyl residue in the flavin binding site of the flavodoxin. Evidence of the presence of such a residue in the FMN binding site of the Shethna Azotobacter flavodoxin is provided from the work of Ryan and Tollin (99).

In their work they observed that one tryptophyl residue was readily reactive with NBS. Upon NBS oxidation of one tryptophyl residue of the

apoprotein they observed a loss of FMN binding ability in the Azotobacter flavodoxin and a 90% reduction of the protein fluorescence. The holoprotein offered similar reactivity to NBS oxidation and was followed by a release of the bound flavin. They conclude the likelyhood of a highly fluorescent tryptophyl residue in the FMN binding site of this flavoprotein which is largely responsible for FMN binding and whose fluorescence is quenched by FMN binding.

Quenching of Flavin and Protein (tryptophan) fluorescence

Upon flavin binding to the flavodoxins both the protein fluorescence and the flavin fluorescence are nearly totally quenched. In at least one flavoprotein (Shethna Azotobacter) one highly fluorescent tryptophyl residue is responsible for the major part of the protein fluorescence and complete quenching of this highly fluorescent tryptophyl residue and the flavin occurs upon flavin binding.

Energy transfer of the Förster type is possible from tryptophan to FMN and could explain in part the fluorescence quenching of this tryptophyl residue by such an excited state process upon FMN binding. However, because of poor spectral overlap between the flavin emission and tryptophyl absorption, energy transfer from FMN to tryptophan is not possible and cannot explain the quenching of FMN fluorescence upon binding of the flavin to the protein.

Ryan and Tollin have determined the rate constants for flavin binding by FMN and protein fluorescence quenching. The similarity of these rate constants lead them to conclude that the same process is responsible for the quenching of both FMN and tryptophyl (protein) fluorescence.

Protein fluorescence lifetime measurements by Andrews et al (105)

have been used to study the binding of FMN to Azotobacter flavodoxin. The fluorescence decay of the apoprotein was seen to consist of two components. One component which constitutes approximately 95% of the fluorescence with a lifetime of approximately 4 nanoseconds and a second minor component with a lifetime of approximately 1 nanosecond. Analysis of the yield data implys that the fluorescence of the major component represents the highly fluorescent tryptophyl residue and is nearly completely quenched upon flavin binding. The resulting unquenched component is due to weakly emitting tryptophyl residues away from the FMN binding site. Analysis of the lifetime data with successive degrees of NBS oxidation shows that the various tryptophyl residues (4 total tryptophyl residues) are oxidized at different rates. Oxidation of the highly fluorescent longlived component occurs most easily in the holoprotein with the subsequent release of FMN. Another tryptophyl residue is oxidized at higher NBS concentrations while denaturation of the protein is necessary to yield oxidation of the two remaining tryptophyl residues. Analysis of the lifetime data lead Andrews et al. (105) to conclude that the observed quenching of tryptophyl fluorescence upon FMN binding cannot be explained in terms of energy transfer from tryptophan to the flavin and must occur at the ground state level. Thus the quenching of both FMN and tryptophyl fluorescence is thought to occur by the same ground state interaction. Ryan and Tollin have suggested that such quenching might result from an interaction between the isoalloxazine ring of the flavin and the indole ring of tryptophan (99). We propose that the ground state complex formed between FMN and tryptophan in which an electron is transfered from tryptophan to FMN to form a stable complex can explain the observed difference absorption spectra, the lowered

oxidation-reduction potential of the flavin and the mutual fluorescence quenching of the flavin and highly fluorescent tryptophyl residue in the FMN binding site of this flavoprotein.

Flavin-tryptophyl quenching due to charge transfer complex formation

The ground state quenching of both FMN and a highly fluorescent tryptophyl residue in the FMN binding site of Azotobacter flavodoxin is explainable in terms of the formation of a non-fluorescent charge transfer complex between this residue and FMN. Slifkin (44) has pointed out that charge-transfer complexes with flavins are generally non-fluorescent and may in this sense act as energy sinks. The formation of such non-fluorescent complexes between FMN and Tryptophan seem a likely explanation for a case such as this where both the flavin and tryptophan fluorescence quenching parallel one another and are believed to result from the same ground state interaction.

Weber (106) first suggested that the interaction of aromatic amino acids residues with flavin could cause quenching of the flavin fluorescence in flavoproteins. If we assume that flavin and tryptophan form a non-fluorescent complex, C, then increasing concentrations of quencher (tryptophan) should cause an increase in the quenching of the FMN fluorescence which is proportional to the association constant K_a of the complex. This quenching can be described by the Stern-Volmer equation $R_0/R - 1 = K_a$ [tryptophan] where R_o is the initial fluorescence without quencher, R the fluorescence after addition of quencher and K_a the association constant of the non-fluorescent complex. In Figure XII we show a Stern-Volmer plot for 2×10^{-5}M FMN and concentrations of tryptophan decreasing from 3×10^{-4}M. K_a was determined to be 120 liters/mole from the slope of this straight line plot. It is not surprising that this

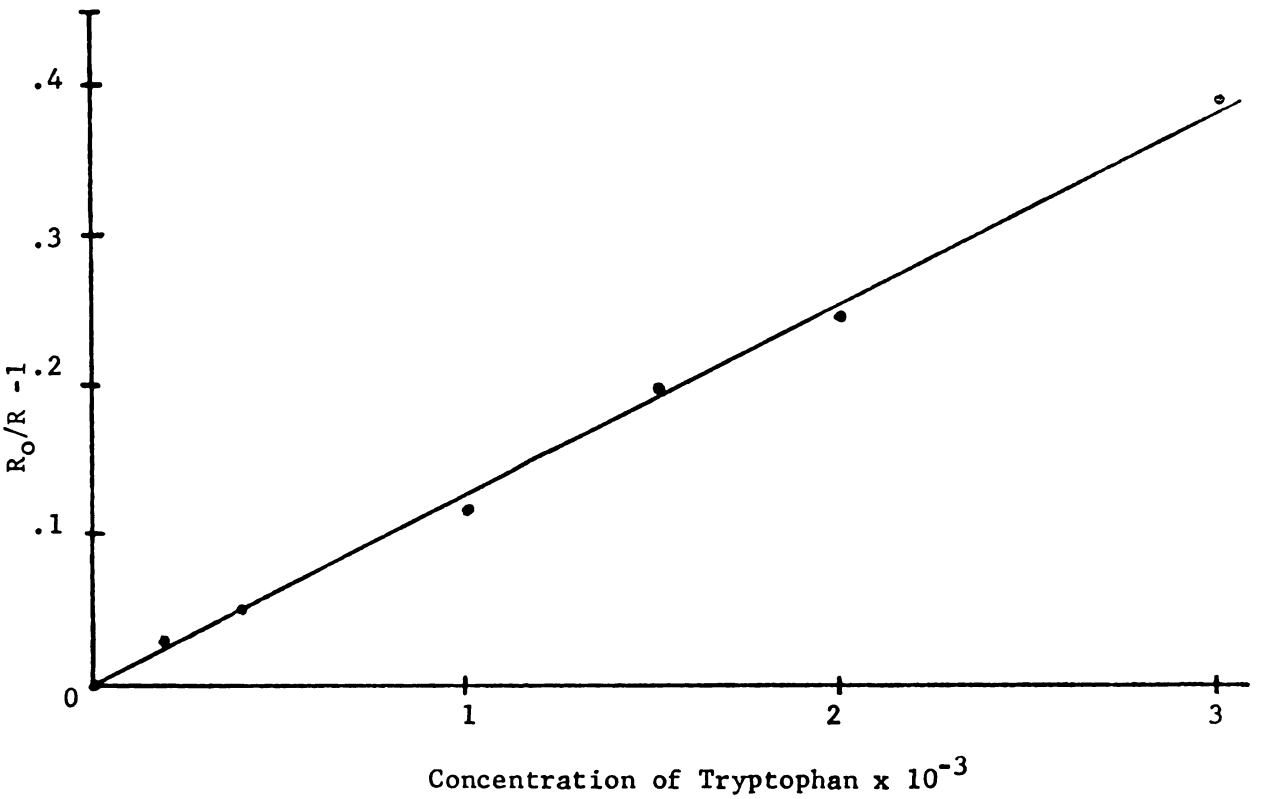


Figure XII.
Stern-Volmer Plot of FMN fluorescence quenching by Tryptophan

fluorescent technique gives a somewhat larger complex association constant than the value of 95 liters/mole that we determined from absorption techniques. The absorbance measurements give only an indication of the strength of the complex in the ground state while association constants derived from fluorescent techniques may contain an additional contribution from an excited state complex (44). The similarity of the two K_a values do however suggest that a non-fluorescent complex is formed in the ground state that results in the quenching of the flavin fluorescence.

Increased quenching of tryptophyl fluorescence is also observed with increasing concentrations of FMN in solution. This quenching does not however yield a linear Stern-Volmer plot due to the fact that energy transfer from tryptophan to flavin is present as an additional means of depopulating the excited state of tryptophan. Also the Stern-Volmer plots in this case are complicated by the interfilter effect since the flavin has substantial absorption in the region of tryptophyl absorption.

Indole and indole derivatives are known to eject electrons to the solvent upon irradiation (107, 110). Such electron ejection is observed to quench the fluorescence of the indole. Feitelson has observed the formation of hydrated electrons from the excited state of indole and the corresponding decrease in its fluorescence (108). Steiner and Kirby have observed fluorescent quenching of indole with the addition of electron scavengers to indole solutions (109). Similarily in a charge-transfer complex between FMN and a tryptophyl residue, the transfer of an electron from the indole ring to the flavin would be expected to quench the fluorescence of that tryptophyl residue very efficiently. Thus the mutual quenching of both flavin and tryptophyl fluorescence can be ex-

plained by the formation of a ground state complex in which an electron is transfered from the tryptophan to the flavin.

We have observed increasing quenching of both tryptophan and FMN fluorescence by increasing the concentration of FMN and tryptophan respectively. An analysis of flavin quenching by complex formation with tryptophan gave an association constant of the same order as that obtained for ground state complex formation. These results indicate that the ground state complex formed between FMN and tryptophan is nonfluorescent and thus complex formation results in the mutual quenching of both flavin and tryptophan fluorescence by the same ground state process.

These results are compatable with the observed quenching of the flavin fluorescence by complex formation with phenols and indoles (47) and the results of Shiga and Piette (111) who have shown that a ground state interaction between FMN and tryptophan in rigid solutions at 77°K quenches the flavin triplet state.

Flavinylpeptides

Some very interesting studies of the absorption, emission and electrochemical properties of synthetic flavinylpeptides have been conducted by Fory, MacKenzie, Wu, and McCormick (59, 112, 113, 114, 115). In these synthetic flavinylpeptides, L-tryptophan, L-tyrosine, and L-phenylalanine were attached to the flavin by a peptide bond through a variable length methylene chain. The absorption spectra of flavinyltryptophan was observed to have a red shoulder off the 450nm flavin absorption band (59, 115). The difference absorption spectra of these flavinylpeptides show positive absorption peaks near 480-490nm. While flavinyltryptophan shows a distinct negative mirror image of the flavin absorption, flavinyltyrosine and flavinylphenylalanine exhibit much less of a negative mirror

image of the flavin absorption. The difference absorption spectra and structure of these flavinylpeptides are shown in figure XIII. These spectra point out however, that while complex formation between tyrosine and phenylalanine with the flavin may be very weak relative to flavin-tryptophan complexes in solution, flavin may complex with tyrosine and to a lesser extent phenylalanine, when held together intramolecularly as in these flavinylpeptides. Thus complex formation could occur with aromatic amino acids such as tyrosine in the flavin binding site of flavoproteins. However with the Azotobacter and Clostridial flavodoxins a tryptophyl residue is clearly involved in close association with the flavin and the observed difference spectra results from flavin-tryptophyl complexation.

In these flavinylpeptides fluorescent quenching of flavin fluorescence is observed for flavinyltryptophan and flavinyltyrosine (114). Maximal and complete quenching of the flavin fluorescence occurs at n=1 for flavinyltryptophan. At n=1 the quenching is due primarily to ground state interactions and is maximal. This shows that a close interaction is necessary for the greatest quenching of flavin fluorescence. As n is increased, quenching by ground state complex formation decreases and excited state (collisional) quenching processes dominate at n=5. Since significant flavin fluorescent quenching occurs also in non-aqueous solvents with flavinyltryptophan and flavinyltyrosine dipole-dipole type of interactions or dispersion forces may be involved in the stabilization of these complexes. Hydrogen bonding between the hydroxyl group of tyrosine and the 4-carbonyl of the flavin may also occur in solvents such as chloroform. In water effective quenching is observed with O-methyltyrosine, implying that H-bonding is not a major stabilizing

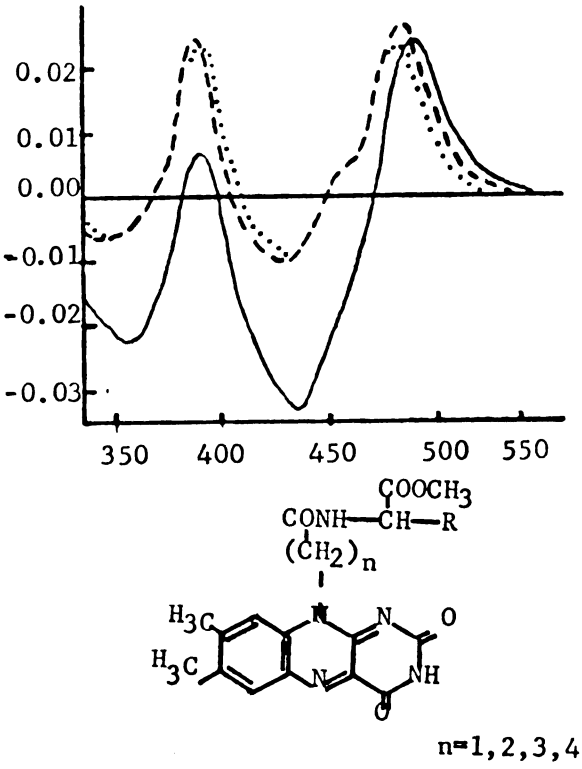


Figure XIII. Difference spectra of flavinyl peptides (n=1) in $5x10^{-2}$M sodium phosphate. pH 7.0. Sample: $2x10^{-2}$M flavinyl peptide; reference: $2x10^{-2}$M N-carboxyalkyl-flavin. Reproduced from Figure 1. reference 112. R= Tryptophan (———), Tyrosine (- - - -) and Phenyl-alanine (·····).

force of the complex in aqueous solvents.

Quenching by ground state complex formation is greatest for flavinyltryptophan. With n=1, 98% of the flavin fluorescence is quenched by the formation of a ground state complex while only 73% of the flavin fluorescence is quenched by such complex formation with n=1 in flavinyltyrosine. With the peptide linked through the N_3 position of the flavin rather than the N_{10} position, no quenching occurs by ground state processes with flavinyltyrosine (n=1) while 74% of the flavin fluorescence is quenched with flavinyltryptophan.

These quenching studies clearly show that a tight ground state complex between the flavin and aromatic amino acids occurs and, for tryptophan, results in nearly complete quenching of the flavin fluorescence. Less flavin quenching by ground state complex formation occurs with tyrosine and even less with phenylalanine. More recent studies by Wu and McCormick (113) have shown that the fluorescence of the aromatic amino acid portions of the flavinylpeptides and the flavin fluorescence are mutually quenched. Here again, quenching is greatest for the tyrptophyl peptides, which are more tightly complexed and least for the phenylalanyl peptides.

These studies with flavinylpeptides show that tryptophan forms the tightest ground state complex with flavin. Resulting from this complex is a red shoulder in the flavin 450nm absorption band and a broadening of this absorption band to the red. There is also a lowering of the oxidation-reduction potential of the flavin which can be explained as a selective decrease in the effective concentration of oxidized versus reduced flavin (42, 115). Mutual quenching of both flavin and tryptophyl fluorescence is seen to occur by the formation of a non-fluorescent ground state complex.

These results from the study of flavinylpeptides as well as the results from spectral studies of FMN-tryptophan solutions indicate that a similar complex between the flavin and a tryptophyl residue of the flavodoxin may occur in some flavoproteins. The formation of a non-fluorescent charge transfer complex between FMN and the highly fluorescent tryptophyl residue in the FMN binding site of the Shethna Azotobacter flavodoxin seems to explain the observed difference absorption spectra which is characteristic of such complex formation, the observed decrease in the oxidation-reduction potential upon flavin binding and the highly efficient quenching of both protein and flavin fluorescence by the same ground state interaction. It is quite encouraging indeed, in terms of such suggestions, to observe the recently determined X-ray structure of D. vulgaris and Clostridial M.P. flavodoxin in the region of the FMN binding site.

The Flavin Binding Site of Azotobacter (D. Vulgaris) Flavoproteins

The detailed X-ray structure of two flavodoxins has now been determined at high resolution. The structure of the D.Vulgaris flavodoxin has been determined by Watenpaugh, Sieker and Jensen (116). Their studies reveal that the FMN moiety is sandwiched between two aromatic amino acids. Tyrosine 98 lies on the solvent side of the flavin and tryptophan 60 lies on the protein side of the flavin sandwich. This geometry is shown in figure XIV. The authors point out that tyrosine 98 lies nearly coplanar (with approximately 15^{o} between the normal to the planes of the rings) and could easily be rotated about the C-C bond to yield coplanarity. In this protein however, it is clear that major changes in the protein structure would be necessary to make tryptophan 60 coplanar with the flavin. For example, as Figure XIV

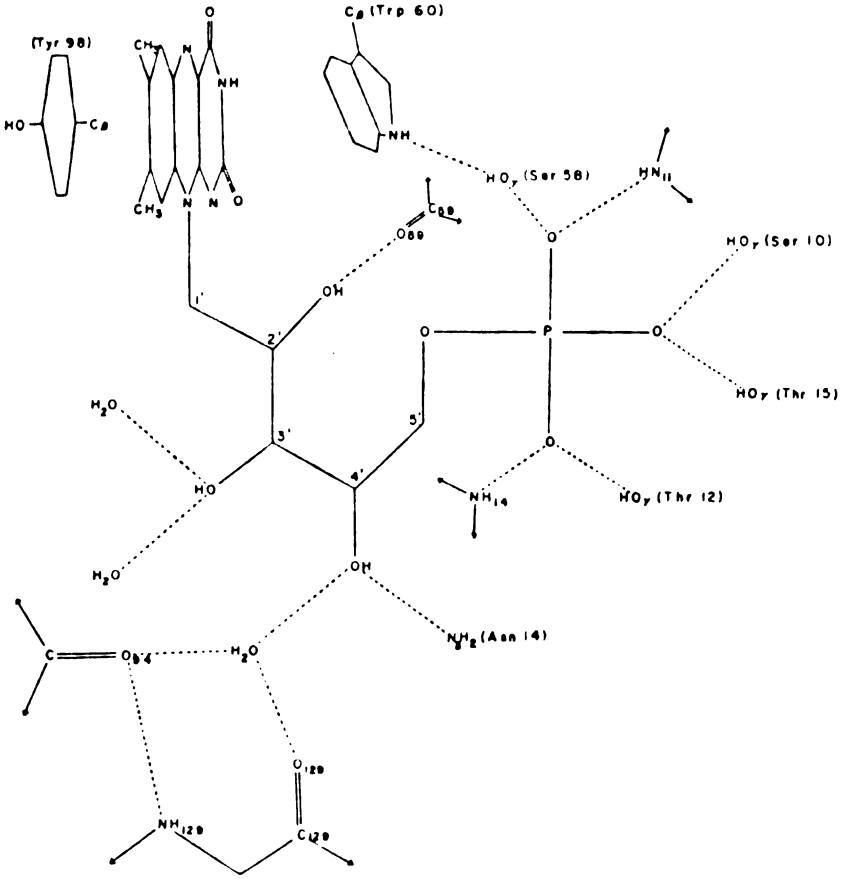


Figure XIV. Geometry of the FMN binding site in D. Vulgaris. Taken from Figure 5 of reference 116.

clearly shows the hydrogen bond between tryptophan 60 and serine 58 would have to be broken to establish coplanarity between the flavin and this tryptophyl residue. It does appear likely, however, that the geometry of the flavin binding site in this flavoprotein is adequate to allow complex formation between the flavin and tyrosine 98.

The Shethna Azotobacter flavodoxin has similar spectral properties as the D. vulgaris protein suggesting that the environment of the flavin in both proteins may be similar. Since in the Azotobacter flavodoxin the quenching of a highly fluorescent tryptophyl residue occurs upon flavin binding or NBS oxidation it is likely that the tyrosine 98 of D. vulgaris may be replaced by a tryptophyl residue in the Azotobacter flavin binding site. Compatible with this idea is the observation that while both D. vulgaris and Azotobacter have five tyrosine residues, the Azotobacter has four tryptophyl residues and D. vulgaris only two. It is also interesting to note that the X-ray structure of the Clostridial flavodoxin as determined by Ludwig et al. at the University of Michigan (117) shows that the tyrosine 98 of D. vulgaris is replaced by tryptophan 90 in the flavin binding site of the Clostridial flavodoxin. If a similar substitution occurs in the Azotobacter flavodoxin it would provide a solvent exposed tryptophyl residue in a geometry appropriate for complex formation with the flavin. This would explain the NBS oxidation properties of this flavoprotein, the loss of fluorescence and FMN binding ability with NBS oxidation of a single tryptophyl residue, the observed absorption and difference absorption spectra of the flavoprotein, the mutual fluorescence quenching by ground state complex formation of the flavin and protein fluorescence and the observed lowering of the oxidation-reduction potential of the flavin upon binding.

The Flavin Binding Site of Clostridial Flavodoxin

Several studies of Clostridial flavodoxins indicate that a tryptophyl residue lies in the FMN binding site and is responsible to a large extent for flavin binding. Oxidation of Clostridial flavodoxin with NBS shows ready oxidation of two tryptophyl residues with the subsequent loss of flavin binding ability and activity (115).

Proton magnetic resonance spectra of Clostridium M.P. and peptostreptococcus elsdenic have been useful in comparing the conformation of the oxidized and reduced forms of the proteins (118). The paramagnetic effects on flavin free radicals is thought to be responsible for the broadening observed with the semiquinone form. The chemical shifts of the paramagnetically broadened lines used in conjunction with X-ray data were used in peak assignment for the amino acid residues near the FMN moiety. Peaks at 5.99 and 6.93ppm (parts per million), which correspond to the aromatic region of the spectra of Cl. M.P. flavoprotein in the oxidized or reduced form, were tremendously broadened in the semiquinone form. X-ray studies of C. M.P. suggest that these two resonances are due to protons of an aromatic residue approximately parallel to the isoalloxozine ring system of the flavin. Higher resolution (1.9Å) X-ray analysis shows this residue to be tryptophan.

Approximation of the peak positions for tryptophan 90 involved in a stacking interaction with FMN yield peak positions at 6.12 to 6.19 ppm and 6.70 to 6.80ppm in rough agreement with the observed peak positions. Similar arguments with P. elsdenic flavodoxin lead to the conclusion that tryptophan 90 is stacked with the isoalloxozine ring system in this protein also (118).

High resolution of the oxidized form of Cl. M.P. flavodoxin has

allowed a detailed description of the FMN binding site in this protein (117). This analysis shows the FMN moiety sandwiched between two residues of the protein, tryptophan 90 and methionine 56. Methionine 56 lies between the FMN molecule and the interior of the protein while the tryptophan 90 residue separates the FMN group from the solvent to a great extent. The ribityl side chain extends away from the ring in towards the center of the protein. In the oxidized form tryptophan 90 and the FMN rings are imperfectly stacked, the normal to the plane of their rings being 27^{o} from parallel. The closest approach is the C_{N} of the tryptophan ring to the N_{10} position of the flavin. The geometry of this flavin binding site is shown in Figures XV and XVI. Rotation about the $C_{\beta} - C_{\gamma}$ bond is easily possible to establish coplanarity of the ring of tryptophan and FMN which is thought to be the necessary geometry for such complexes (114, 119, 120, 121). The nearness of tryptophyl 90 to the flavin in the flavin binding site of this flavodoxin suggests the high probability that complex formation between this tryptophyl residue and the flavin might occur. Thus we see that in at least three flavoproteins Azotobacter, Clostridial M.P., and P. elsdenic tryptophyl residues have been implicated in very close association with the flavin group.

Summary of Complex Formation in Flavodoxins

In summary, we see that FMN-tryptophan solutions, flavinylpeptides of tryptophan and at least three flavoproteins with bound flavin all have common spectral and electrochemical properties. In each there is a broadening of the long wavelength flavin absorption band relative to that of the free flavin with a red shoulder which shows clearly as a difference absorption maxima near 490 to 500nm. In each, the extinction coefficients of the flavin absorption is diminished and the oxidation-reduction potential of the flavin is decreased. These latter observations are consistent

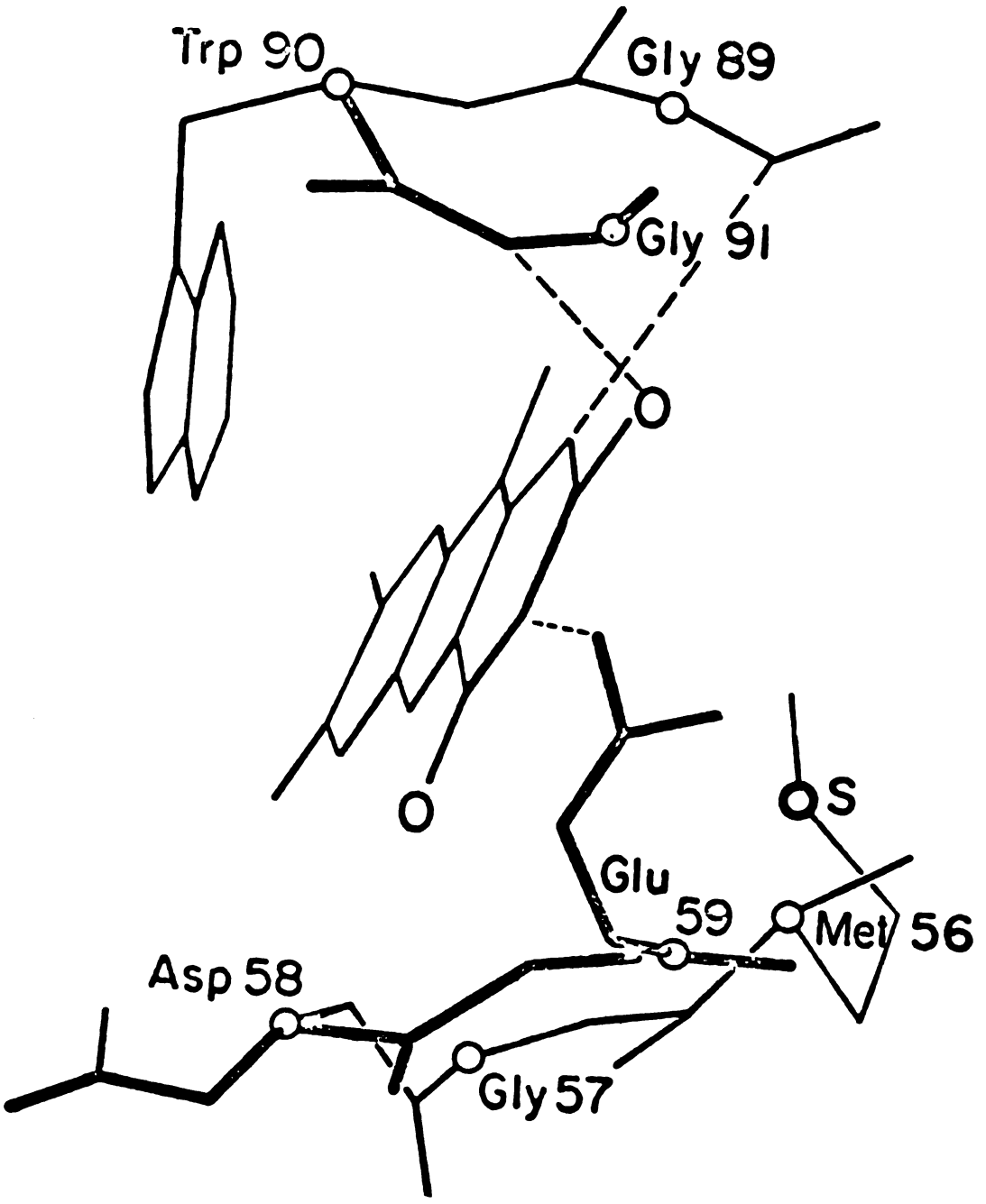


Figure XV. Orientation of flavin and tryptophan 90 in FMN binding site of Cl. M.P. taken from reference 117.

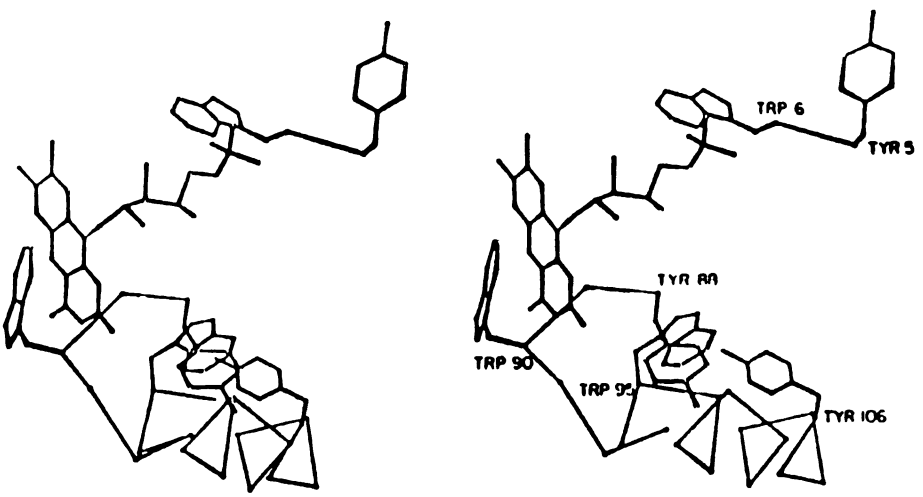


Figure XVI. Geometry of the Flavin Moiety in the FMN binding site of Cl. M.P. taken from reference 117.

with a selective increase in the amount of reduced flavin relative to oxidized flavin. Also, in each, the fluorescence of the flavin and tryptophan are both mutually quenched by the formation of a non-fluorescent ground state complex between the flavin and tryptophan.

In FMN-tryptophan solutions these phenomena arise from the formation of a complex between the oxidized flavin and tryptophan in which an electron is transfered from tryptophan to the flavin. This complex stabilizes the partially reduced form of the flavin in such solutions. The previous work of many investigators and in some cases high resolution X-ray analysis have shown the presence of tryptophyl residues in very close association with the flavin in Clostridial M.P., Azotobacter and P.elsdenic flavodoxins. These facts considered together suggest to us the likelihood that a similar complex occurs between a tryptophyl residue in the FMN binding site and the flavin in at least these three flavoproteins.

Even though there exists a wealth of evidence indicating the presence of a tryptophyl residue in the FMN binding site, investigators have not been open to the fact that FMN and tryptophan are capable of charge transfer complex formation. Consequently they have not recognized the charge transfer nature of the complex formed between FMN and a tryptophyl residue in the FMN binding site of some flavodoxins. We present for the first time absorption spectra, difference absorption spectra, electrochemical data and flavin, tryptophan fluorescent quenching data as evidence that such complex formation occurs in several flavodoxins. This evidence should encourage investigators to view the FMN-tryptophan complex in flavodoxins as a strong complex between the most efficient electron donating amino acid, tryptophan, and the excellent electron

acceptor, FMN, through which an electron is nearly completely transfered from the indole ring to the flavin prosthetic group. The formation of such specific complexes will not only explain the observed spectral and electrochemical properties of the flavodoxins but should lead investigators to a better understanding of the mechanism of flavodoxin action and perhaps electron transport in general.

The Biological Significance of Charge Transfer

We have concerned ourselves with the complex formed between tryptophan, the most efficient electron donating amino acid, and FMN, an excellent electron acceptor. In the complex that these molecules form, an electron is transfered from the indole ring of tryptophan to the isoalloxazine ring of the flavin. Upon complex formation a new absorption appears on the red edge of the 450nm flavin absorption which is clearly visible as a difference absorption maxima near 500nm. This new absorption is characteristic of the flavin semi-reduced form and indicates that electron transfer has occured. Although this complex may be referred to as a charge transfer complex the new absorption is not a charge transfer absorption band but rather the absorption from a new molecular species, the flavin semiquinone which is produced by complex formation and electron transfer.

Since the initial studies of charge transfer interactions by Mulliken, many investigators have realized the potential biological significance of such phenomena. A. Szent-Gyorgyi has suggested that charge transfer interactions may be one of the most important, frequent and fundamental biological reactions (53). Despite very little evidence for charge transfer complexes in biological mechanics, the beauty and the promise of such interactions in explaining biological phenomena have

kept many investigators searching for examples of charge transfer in nature.

The most obvious property of such complexes is the transfer of charge between molecules. Electrons of charge transfer are obviously of vital importance in biology. Charge transfer interactions seem promising in explaining electron transport and biological oxidation and reduction processes since charge transfer interactions allow the transfer of an electron between two substances without the necessity of a dramatic rearrangement of molecular structure. It is also interesting to note that charge transfer interactions allow the transfer of an electron between two substances over a relatively long distance (3 to 3.5Å) compared to chemical bonds which are generally less than 1.5Å. In this sense charge transfer complexes could allow interactions over relatively long separations.

Through charge transfer interactions two previously inactive molecules may become activated and highly reactive. When the donor molecule transfers an electron to the acceptor the resulting hole in the ground state level of the donor molecule makes it a very good electron acceptor. Similarly, the acceptor after it acquires an electron is capable of functioning in a new role as an electron donor. Thus two molecules that were initially relatively unreactive are now primed for subsequent reactions by charge transfer and become reactive species with definite chemical functions. In perhaps a more extreme case, complex dissociation may occur and two very reactive free radicals are formed.

Some authors have used charge transfer complex formation to open the door for semiconduction in biology. If the acceptor acquires an electron from a saturated energy band, a hole is created, and this band may become

conductant. Similarly, if a donor transfers an electron to a previously empty energy band it may then become conductant.

Charge transfer forces may also serve to contribute to the association of two or more molecular species. A special or preferred conformation may result which in turn may confer biological activity to these species. This new association will obviously influence the equilibrium and reactivity of certain chemical species. For example, Cilento and Berenhole (122) have observed that differences in the complexing abilities of the ionized and neutral forms of a molecule with certain species changes the pK value.

Despite all of these properties that charge transfer complexes possess which could be very important in biological processes it is perhaps an unhappy consequence of our limited understanding of nature that there is no clear evidence of any role played by charge transfer complexes in biological systems.

Perhaps the most likely place to look for such complex formation and their biological role is the small flavodoxin proteins. Flavodoxins are interesting both as models for the action of the much larger flavoproteins as well as for their own properties. They serve to form a shuttle system for electrons. They accept, carry and donate electrons back and forth between the appropriate proteins. The flavin cofactor in these proteins serves as the electron collection point from various substrates. It can be partially or fully reduced by accepting one or two electrons respectively and can then donate one or both of these accepted electrons and become oxidized.

No report of charge transfer association between FMN and any flavoprotein has yet been demonstrated. The evidence that we have pre-

sented clearly suggests that such complex formation occurs between a tryptophyl residue in the FMN binding site of several flavodoxins and the FMN group itself.

While the exact role of such complex formation in flavodoxins is uncertain and thus open to speculation, the formation of such a specific complex may play a very significant role in the actions of these flavoproteins. The knowledge that such complex formation occurs and that electrons are transfered from tryptophan to the flavin should prove very helpful in advancing the understanding of the mechanism of flavoprotein action.

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