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A KINETIC STUDY OF FLAVINS AND FLAVOPROTEINS

by

Bruce George Barman

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF CHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1972
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ABSTRACT

The kinetic mechanisms associated with partially reduced flavin solutions have been investigated and found to be consistent with earlier proposals in which a complex of oxidized and fully reduced flavin was suggested as a precursor to the flavin semiquinone. Measurement of the rates of semiquinone disproportionation by temperature-jump relaxation and flash photolysis methods differed considerably (two orders of magnitude). It was found that this is due to the formation (at the high flavin concentration used in the temperature-jump experiments) of a complex between oxidized and reduced flavin that contains considerable radical dimer character. The complex is best represented as an equilibrium mixture of a charge transfer complex and a radical dimer with about equal contribution from each. This complex was isolated in solid form and found to contain from 30-50% unpaired spin concentration per flavin molecule.

The kinetics of flavin complexation with several aromatic compounds (i.e., tryptophan, tyrosine, serotonin, etc.) of biological significance were explored. It was found that in all cases the bimolecular complexation rate constants were greater than $10^7 \text{ M}^{-1}\text{sec}^{-1}$. This indicates that flavin complexation with aromatic residues in flavoproteins is not rate limiting for the binding of the coenzyme to the protein.
Flavin-protein interactions have been investigated using the Shethna flavoprotein from *Azotobacter vinelandii*. The temperature-jump relaxation method was used for determining the kinetics of binding of several flavin analogs to the Shethna apoprotein. Analogs were chosen such that the importance of ribityl phosphate side chain and N-5 isoalloxazine ring positions were explored. The results indicate that the phosphate group induces a conformational change in the protein subsequent to the initial binding process. This conformational step was absent in nonphosphorylated analogs. The thermodynamics and equilibrium and rate constants for analog binding were also determined.

The reduction properties of free 5-deazaflavins and the 5-deazaFMN-Shethna apoprotein complex were investigated. It was found that the substitution of carbon for nitrogen at the 5-position did not qualitatively change the reduction properties of the free flavin molecule. However, substitution at this position in the protein complex completely destroyed the ability of the flavoprotein to form a semiquinone, thus indicating the presence of an important flavin-protein interaction at this position.

The reduction potentials for the Shethna flavoprotein were also measured ($E_1^o = +50$ mv; $E_2^o = -495$ mv). The second reduction potential is the most negative of all biological redox enzymes and is consistent with the flavodoxin activity of this protein. The kinetics of the flavoprotein semiquinone/hydroquinone equilibrium were determined by the temperature-jump method. No conformational steps were found to be associated with this equilibrium, which is consistent with earlier CD
studies. The reduction potential was calculated from the kinetic analysis and found to agree well with the $E^\circ_2$ determined by potentiometric titration.
INTRODUCTION

Flavins and flavoproteins have been found to play an increasingly important role in biochemistry ever since Warburg and Christian (1932) discovered the "old yellow enzyme," and Theorell (1935) showed that the yellow color of this enzyme was due to the 5'-phosphorylated derivative of the vitamin riboflavin. Since that time, a great many other flavin-containing proteins have been isolated and shown to be important in catalyzing biological oxidation-reduction reactions. The flavoproteins have been found to function in a wide array of metabolic processes, such as respiration, photosynthesis, and amino acid and fatty acid metabolism (Mahler and Cordes 1966).

The flavin coenzyme has great versatility as a redox agent due to its ability to participate in one or two electron transfer processes. A thorough knowledge of the properties of free flavin is necessary in order to more fully understand the chemistry of flavoproteins. In addition, it is of considerable importance to compare these properties with those of flavoproteins in order to understand the nature of the flavin-protein interactions and how these act to modify flavin chemistry. Such studies may also be relevant to coenzyme-protein interactions in general.

The purpose of the research reported in this dissertation was to investigate some aspects of flavin-protein interactions by studying (1) the redox equilibria of free flavins, (2) the complexation
behavior of flavins, (3) the effect of flavin modification upon binding to protein, (4) the effect of flavin modification on the redox properties of a flavoprotein, and (5) the kinetics of reduction and redox potentials of a native flavoprotein.

This study was greatly facilitated by the recent isolation of the flavodoxins, which are relatively simple flavoproteins (MW = 10,000-30,000) containing only a single flavin of cofactor. The work presented herein is primarily concerned with the Shethna flavoprotein of Azotobacter vinelandii, which is of the flavodoxin type.

Of the two important coenzymes occurring in nature, only flavin mononucleotide (FMN) is dealt with in this study. Thus only the properties of this coenzyme and some of its derivatives will be discussed (Figure 1).

The flavin coenzyme is capable of participating in both one and two electron transfer processes. Therefore, the fully oxidized, half-reduced, and fully reduced flavin are of biological interest. Some of the important properties of these three forms are subsequently presented. A more detailed discussion of the spectral and ionization properties of flavin in its various redox forms has been given by Edmondson (1970).

**Flavins**

**Properties of Oxidized Flavins**

Fully oxidized FMN has strong absorption peaks at 375 nm \( (E = 10,600 \, \text{M}^{-1}\text{cm}^{-1}) \) and 445 nm \( (E = 12,012 \, \text{M}^{-1}\text{cm}^{-1}) \) (Beinert 1960) in the visible spectral region. Flavin mononucleotide also exhibits an
Figure 1. Structures of flavin derivatives involved in this study.
intense fluorescence centered about 530 nm. In water, the flavin derivatives riboflavin and lumiflavin (Figure 1) show almost identical absorption and fluorescence behavior as does FMN. The effect of solvent on the spectral properties of flavins has been discussed elsewhere (Koziol 1969). In addition, the effect of substitution on the isoalloxazine ring on the absorption behavior of flavins has been presented in detail by Penzer and Radda (1967).

In addition to ordinary absorption spectra, FMN and riboflavin, as well as some of their analogs, show optical activity due to the asymmetry of the ribityl side chain. Edmondson and Tollin (1971, p. 124) have used this property in circular dichroism (CD) studies to probe the flavin environment in some of the flavodoxin proteins.

Oxidized flavins are good complexing agents with a wide variety of aromatic molecules (Penzer and Radda 1967, Tollin 1968). Among these are phenols (Fleischman and Tollin 1965), indoles such as tryptophan and serotonin (Isenberg and Szent-Gyorgi 1958), purines and pyrimidines (Radda and Calvin 1964), tyrosine (Harbury and Foley 1958), and other flavin redox states (Gibson, Massey, and Atherton 1962).

There is still some controversy as to the forces involved in stabilizing these complexes. Besides the usual vander-Waals and hydrogen bonding attractions, it is believed that charge-transfer interactions (Mulliken 1952) also occur (Harbury and Foley 1958). However, many of these complexes do not show a separate charge-transfer absorption band and Weber (1966) feels that ground state charge-transfer is not necessarily important (cf Tollin 1968).
Nearly all of the complexes show an intensity decrease (hypochromism) in the flavin absorption at 375 nm, accompanied by both a broadening and a decrease in the 445 nm band, such that the long wavelength edge exceeds that of free flavin (Weber 1966, Fleischman and Tollin 1965). This is especially interesting when one considers that similar effects are observed when flavins are bound to proteins. The fact that flavins form complexes with tyrosine and tryptophan indicates that such complexation may be important in the flavin-protein interaction. In this regard, it is also interesting that flavin fluorescence is quenched by both complexation and binding to proteins.

It may also be significant that flavins complex with reduced pyridine nucleotides (NADH), inasmuch as certain flavoproteins are known to oxidize these compounds. This suggests that the first step in reduction of the flavoproteins may be complexation with the reducing agent (Massey and Veeger 1961).

Another feature of flavin complexation is that water appears to be an indispensable constituent for stabilization (Weber 1966, Tollin 1968). This raises the possibility that water may be involved at the flavin binding site within flavoproteins.

Properties of the Flavin Semiquinones

The existence of a flavin semiquinone has been confirmed by ESR studies on partially reduced flavin solutions (Beinert 1960). The formation of such a species was originally suggested by Kuhn and Wagner-Jauregg (1934) while studying the reduction of riboflavin by zinc and acid.
The flavin radical can exist in two biologically important states of protonation: a neutral and an anionic form. The pK of the ionization of the neutral semiquinone to its anion form has been determined to be 8.3 for riboflavin and 8.5 for FMN (Draper and Ingraham 1968, Land and Swallow 1969, Vaish and Tollin 1970). The equilibrium between the two forms is illustrated below.

Several different tautomeric forms are available to each of the species. The neutral radical is believed to give rise to blue semiquinone flavoproteins while the anion radical gives rise to red semiquinone flavoproteins (Massey and Palmer 1966). The absorption spectra of the neutral and anion radicals have been determined by pulse radiolysis spectral studies (Land and Swallow 1969) and differ appreciably from oxidized flavins. For the neutral radical there are peaks at 340 (\(E = 10,500 \text{ M}^{-1}\text{cm}^{-1}\)), 510 (\(E = 5,000 \text{ M}^{-1}\text{cm}^{-1}\)) and 580 nm (\(E = 4,700 \text{ M}^{-1}\text{cm}^{-1}\)). For the anion radical there are peaks at 470 (\(E = 3,000 \text{ M}^{-1}\text{cm}^{-1}\)) and 260 nm (\(E = 16,000 \text{ M}^{-1}\text{cm}^{-1}\)). In addition to the large change in absorption, the flavin fluorescence is also lost for both forms of the semiquinone.

Using potentiometric titrations, Draper and Ingraham (1968) have determined the reduction potentials for the oxidized/semiquinone FMN pair in the pH range 3 — 13 (\(E^* = 0.0146 \quad -0.498\)).
The semiquinone flavins also form complexes with aromatic molecules (Draper and Ingraham 1970). In fact, there is enhancement in the association constants for complex formation over that of oxidized flavin. Interestingly, Mayhew (1971a) has found an enhancement in the binding of the flavin semiquinone to the apoprotein of *P. elsdonii* flavodoxin. As will be shown below, we have found a similar effect with the Shethna flavoprotein.

The semiquinone state may be produced by either chemical (Beinert 1960) or photochemical reduction (Massey and Palmer 1966). In the chemical method, a less than stoichiometric amount of sodium dithionite is used to achieve partial reduction. In photochemical reduction, flavin solutions are irradiated in the presence of EDTA. The photochemical reduction most probably proceeds via the flavin triplet state (Vaish and Tollin 1970, 1971).

The flavin semiquinone in both its neutral and anion forms undergoes rapid disproportionation to form oxidized and fully reduced flavin (Holmstrom 1964). The rate constants for disproportionation for the FMN neutral and anion radicals have been determined to be $2.6 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$ and $1.0 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$, respectively (Vaish and Tollin 1971). For riboflavin, a somewhat smaller rate constant ($k = 4 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$) was measured by Holmstrom (1964). Swinehart (1965), using the temperature-jump method, calculated the disproportionation rate constant of the FMN neutral radical to be $6 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$. The probable reason for this large discrepancy is resolved in this study.
It has also been found that the rate of oxidation of the flavin semiquinone is highly dependent upon the state of ionization. The anion radical reacts rapidly with oxygen ($k = \sim 10^9$), while the neutral radical is essentially unreactive (Vaish and Tollin 1971). It is interesting that the blue semiquinone dehydrogenases, which are characterized by the presence of the neutral flavin radical, are also very slowly oxidized by oxygen, whereas the red semiquinone oxidases, which contain the anion radical, are very rapidly oxidized by oxygen.

Electron spin resonance studies on free flavin semiquinones have indicated a high spin density at the N(5), N(10), and C(8) positions of the isoalloxazine ring (Guzzo and Tollin 1964; Ehrenberg, Muller, and Hemmerich 1967). This suggests that these positions may be points for electron transfer in flavoprotein semiquinones.

**Properties of Fully Reduced Flavins**

The visible absorption spectrum of fully reduced flavin is markedly different from either the oxidized or semiquinone forms. In the region beyond 300 nm, there is only one peak ($\sim 400$ nm) which tails off slowly to around 480 nm (Beinert 1960). The fully reduced form shows no fluorescence.

Beinert (1960) discovered two absorption bands (at 580 nm and 900 nm) associated with the formation of the flavin semiquinone and showed that ESR signals correlated with these. However, Gibson et al. (1962) found that the 900 nm band varied with concentration in a manner distinct from that of the 580 nm band. They assigned the 900 nm absorption to a charge-transfer complex of reduced and oxidized flavin,
whereas Beinert had assigned this band to a dimer of the flavin semi-quinone. Gibson et al. (1962) also proposed a scheme whereby mixing oxidized and reduced flavin first forms the above mentioned charge-transfer complex which then leads to the formation of the flavin semi-quinone as shown below.

\[
F + FH_2 \xleftrightarrow{\text{[F - FH}_2\text{]}} \xrightarrow{\text{2FH}_2} \]

It was through the use of this mechanism that Swinehart (1965) calculated the rate constant for disproportionation of the flavin semiquinone from temperature-jump measurements. The existence of this charge-transfer complex has been generally accepted, but with some caution because of the tendency of flavins to dimerize in any oxidation state and the lack of information on the true spectrum expected for a flavin-radical dimer.

Draper and Ingraham (1968) have determined the reduction potential for the flavin semiquinone/fully reduced pair in the pH region 3 \(\rightarrow\) 13 (\(E^0 = 0.040 \rightarrow -0.4280\)). They have also determined the association constants for complexation of the fully reduced flavin at pH 5.0 with a number of aromatics and found these constants to be slightly smaller than those for the same complexes with oxidized flavin (Draper and Ingraham 1970). This is not surprising inasmuch as the complexes of flavin with aromatics are probably of the stacked planar type (Fory, MacKenzie, and McCormick 1968). An out-of-plane bending would probably cause a weakening of the forces of complexation. This same effect might be expected in the association of fully reduced flavin with apoprotein.
Usually when the biological redox behavior of flavins is considered, it is presumed that flavin is protein bound and that one is in reality talking about the redox behavior of the holoenzyme. However, there is an example in the bioluminescent reaction of a marine bacterium, Photobacterium fischeri, in which the redox behavior of free flavins is important (Gibson et al. 1966). In the light-emitting enzyme system of this organism, it is found that only the fully reduced flavin (FMNH₂) is bound effectively to the enzyme luciferase and that during the reaction oxidized FMN is released. Therefore, a knowledge of the properties of free flavin is important in this particular case.

One other example of a possible biological functioning of free flavins can be found in an observation by Edmondson and Tollin (1971, p. 133). It was found that the rate of photoreduction of the Shethna flavoprotein is dramatically enhanced by the addition of FMN. A similar effect has been observed with D-amino acid oxidase (McCormick, Koster, and Veeger 1967). Inasmuch as free flavins are present in vivo, it is possible that they may act in a catalytic role in the reduction of flavoenzymes.

Flavoproteins

Properties of Flavodoxin-type Flavoproteins

The flavodoxin-type proteins belong to a larger general class of flavoproteins called the dehydrogenases (Massey et al. 1969). The enzymes in this class are characterized by the high reactivity of their semiquinone forms with one-electron acceptors [eq. Fe(CN)₆⁻³] and their
slow reactivity with \( O_2 \). In addition, the hydroquinone forms of this class are extremely reactive with \( O_2 \). The flavodoxins have additional properties that allow for subclassification. All of the flavodoxins are low molecular weight proteins (10,000 - 30,000) with one FMN per molecule as the prosthetic group and can substitute for ferredoxin in photosynthetic NADP\(^+\) reduction. In addition to these chemical properties, Edmondson and Tollin (1971, p. 113, 124) found similar CD spectra among the flavodoxins and suggested the use of CD as a means of flavoprotein classification. They also found that differences in the redox properties among the various flavodoxins correlate well with small differences in the structure of the CD spectrum suggesting the existence of subgrouping within the flavodoxins. This has been confirmed and extended to other properties by D'Anna and Tollin (1972).

Until recently, the Shethna flavoprotein was not considered to be a flavodoxin, inasmuch as earlier work (Hinkson and Bulen 1967, Benemann et al. 1969, Cusanovich and Edmondson 1971) had failed to show any catalytic activity. However, Van Lin and Bothe (1972) have recently demonstrated using improved methodology that the Shethna flavoprotein can indeed replace ferredoxin in the photosynthetic NADP\(^+\) reduction. Thus, this behavior along with its other chemical properties (Hinkson and Bulen 1967; Edmondson and Tollin 1971, p. 113, 124, 133) indicates that this protein should be classified with the flavodoxins.

The flavodoxins are commonly named for the organism from which they are isolated (i.e., Shethna flavoprotein = \textit{Azotobacter} flavodoxin).
Flavodoxins have been isolated from *Clostridium pasteurianum*, *Clostridium MP.*, *E. coli*, *P. elsdenii*, *Desulfovibrio vulgaris*, *Rhodospirillum rubrum*, *Anacystis nidulans*, *Chlorella fusca*, and *Ankistrodesmus braunii*. With the exception of the *Azotobacter* and *E. coli* enzymes, all of the other flavodoxins are produced only when the organism is grown in an iron-deficient medium. This is interesting because it demonstrates a genetic adaptability of both photosynthetic and nonphotosynthetic bacteria to compensate for deprivation of a necessary mineral. Inasmuch as the *Azotobacter* flavodoxin is constitutive rather than induced, it would be of interest to know its biological role, especially with the knowledge that the organism also produces a ferredoxin. However, work in this area has proven difficult and no clear role is as yet known.

The flavodoxins, because of their dehydrogenase activity and their ability to replace ferredoxin, are believed to shuttle between the hydroquinone and semiquinone states in catalysis. This necessarily implies that the reduction potentials of the flavodoxins must approximate the reduction potential of the ferredoxins (≈ -400 mv). This has been found to be true in the potentiometric studies to date on several of the flavodoxins (Mayhew 1971b, p. 276; Mayhew and Massey 1969; Vetter and Knappe 1971; Van Lin and Bothe 1972). A listing of these reduction potentials is presented later in this text.

Flavin-protein Interactions

A great deal of effort has gone into attempts to elucidate the nature of the flavin-protein interaction. These studies are
facilitated by the fact that in nearly all flavoenzymes the flavin is not covalently bound to the protein and may be resolved by treatment with acid (Warburg and Christian 1938, Hinkson 1968) or high salt (May-hew and Massey 1969). The resolved apoprotein may then be recombined with modified flavins and the enzymatic activity (when possible), redox behavior, and spectral properties compared with the native holoenzyme. In this way, the positions on the isoalloxazine ring and ribityl side chain that influence the above properties can be determined.

It has been found by Tsibris, McCormick, and Wright (1966) that the N(3) position on the isoalloxazine ring is important in that only partial restoration of catalytic activity is obtained when 3-methyl FMN is bound to the apoenzymes of several FMN-containing flavoproteins. A similar requirement for an unsubstituted N(3) position is found in several FAD-containing enzymes (Chassey and McCormick 1965). Edmondson and Tollin (1971, p. 124) have shown that the N(3) position is indicated as a possible hydrogen bonding site within the Shethna protein, inasmuch as methylation of this site in FMN decreases the binding free energy by about one kilocalorie. They have also demonstrated a cooperativity between the 5'-phosphate group of the ribityl side chain and the N(3) position in that 3-methylFMN is bound strongly whereas 3-methyl riboflavin does not bind at all. In addition, the stability of the semiquinone to dithionite reduction is lost upon 3-methylation.

The N(5) position of the isoalloxazine ring, which has been shown to be important because of its high spin and charge density in the free flavin semiquinone, has not been previously studied in
flavoenzymes. With the use of 5-deaza analogs of FMN and riboflavin, the role of this position is explored in the present work.

The hydroxy1 groups of the ribityl side chain have been shown to have little effect on the flavin-protein association constants or the dithionite stability of the semiquinone for the Shethna flavoprotein. However, the removal of these hydroxyls greatly enhances the rate of reduction of the holoenzyme, which suggests that they may provide a steric restriction to reduction (Edmondson and Tollin 1971, p. 124, 133).

It has also been observed that the 5'-phosphate group greatly enhances the flavin-protein association constant for the Shethna flavoprotein (Hinkson 1968; Edmondson and Tollin 1971, p. 124). This same effect is found for the old yellow enzyme (Åkeson, Ehrenberg, and Theorell 1963). In addition, Mayhew (1971b, p. 289) has observed that the ribityl phosphate group is essential to flavin binding in the Clostridial MP and P. elsdenii flavodoxins and D'Anna and Tollin (1972) have suggested that the phosphate group triggers a crucial binding site modification in these proteins.

Edmondson and Tollin (1971, p. 133) have found that the photo-reducibility of bound FMN analogs with the Shethna apoprotein is quite sensitive to the presence or absence of side chain hydroxyl groups and to modification in the pyrimidine ring, but less sensitive to modification in the benzenoid portion of the isoalloxazine ring system. Of considerable importance is that removal of the phosphate of FMN to form
riboflavin completely destroys the semiquinone stability of the Shethna flavoprotein (Edmondson and Tollin 1971, p. 133).

Although little is presently known about the groups in the protein that are responsible for the binding of flavins, some progress has been made in this direction. Because of the complexation behavior of flavins with tryptophan and tyrosine, it has been inferred, as previously mentioned, that this same complexation is responsible in part for flavin binding. Hinkson (1968) has found that nitration of four of the five tyrosines of the Shethna apoprotein eliminated 90% of the FMN binding capacity. Similar work, involving iodination of the tyrosines of the old yellow enzyme apoprotein, gave the same behavior (Åkeson et al. 1963). Edmondson and Tollin (1971, p. 133) found a change in the tyrosyl ionization properties of the Shethna apoprotein upon flavin binding and suggested that this indicates the possibility of tyrosyl residues at or near the flavin binding site.

This implication of tryptophan in the flavin binding process is not as clear. D'Anna and Tollin (1971) found that tryptophan fluorescence is quenched upon flavin binding to the Shethna apoprotein and that the rate of quenching parallels the flavin fluorescence quenching. However, on the basis of excitation spectra they have suggested that there may not be a direct flavin-tryptophan interaction but rather an interruption of tyrosyl-mediated energy transfer. D'Anna and Tollin (1972) have also noted that protein tryptophan fluorescence is quenched upon the binding of FMN to the flavodoxin apoproteins of C. pasteurianum, P. elsdenii, D. vulgaris, and R. rubrum. They have suggested
that the quenching of protein fluorescence indicates that tryptophan either becomes incorporated into the protein interior due to a conformational change or is in close proximity (<10 Å) to the flavin such that energy-transfer quenching occurs. It has also been observed (D'Anna and Tollin 1972) that the flavodoxins which have tryptophan side chains more accessible to solvent (Eisinger 1969, Longworth 1968) bind FMN more slowly.

Application of Relaxation Methods to Flavin-protein Interactions

To date, all of the studies concerning flavin-protein interactions have used static methods such as U.V., fluorescence, and C.D. spectroscopy of analog, holo, and apo flavoproteins, titration methods, equilibrium analog-binding studies, and simple kinetic measurements of rate limiting steps in analog binding. As mentioned previously, some of this work has led to the belief that the phosphate group of FMN induces a protein rearrangement that facilitates the binding process. However, there is no kinetic evidence to support this mechanistic proposal.

With the advent of relaxation methods (Eigen 1954) chemists were given a powerful instrument for the study of rapid biological reactions that are mechanistically complicated. Although the method is relatively new, there has been considerable progress made in determining complicated enzyme kinetics. Most of the studies to date have investigated ligand binding and resultant conformational changes in enzymes. These studies have answered such questions as: What is the
What is the functional role of conformational changes in bringing about specific binding and catalytic activity? Good reviews on many of the enzymes studied have been given by Eigen (1968) and Hammes (1968).

The principle behind relaxation methods is simple. Chemical equilibrium is a dynamic state, dependent upon external variables (e.g., temperature, pressure, etc.), such that any alteration of an external variable will result in an equilibrium shift. In the temperature-jump relaxation method, a reacting system that has reached equilibrium is perturbed by rapidly raising the temperature. The reacting system will subsequently shift to a new equilibrium position characterized by the new temperature. The kinetics involved in going to the new equilibrium will be the same as if reactants had been initially mixed at the elevated temperature. The relaxation approach has the advantage that the perturbation is extremely fast (<10 μ sec) and so very rapid reactions may be followed that could possibly not be seen due to the slowness of mixing. A disadvantage is that the relaxation method requires that an equilibrium must exist between reactants and products in order for the perturbation to have an effect.

For a small perturbation of temperature, reactants will approach their new equilibrium values at the higher temperature exponentially; that is, the rate at which the concentration of a reactant changes is proportional to the difference between its equilibrium concentration and its actual concentration. The reciprocal of the proportionality constant has the units of time and is called the relaxation
time. In other words, it is the time required for the concentration of a reactant to reach 1/e of its new equilibrium value.

The relaxation time is related to the equilibrium concentrations and rate constants for a reacting system and its exact form depends on the reaction mechanism. For each independent step in a reaction mechanism there will be a separate relaxation. Therefore, a knowledge of the individual relaxation times and their concentration behavior will allow the determination of the kinetic parameters and steps characterizing a reaction mechanism. This makes relaxation methods considerably more powerful than rapid mixing techniques inasmuch as the latter can only provide information concerning the rate-limiting step in a sequence.

It should be possible by applying the temperature-jump method to the binding of flavin analogs to the Shethna apoprotein to obtain the mechanism of binding. Any conformational steps subsequent to flavin binding should be observed as separate relaxation effects that are concentration-independent. The results of experiments of this type are presented in the following section. In addition, the method of temperature-jump relaxation spectroscopy may be applied to free flavin redox equilibria, the reduction of flavoproteins, and the complexation of flavins with aromatics. The results of these experiments are also presented in this dissertation.
EXPERIMENTAL

Materials

Flavin Analogs

Riboflavin (Rbf) and flavin mononucleotide (FMN) were obtained from Calbiochem, Los Angeles, California. Riboflavin was used without further purification. Flavin mononucleotide was purified by DEAE-cellulose column chromatography at pH 7.0. Dr. C. C. Chang, Midwest Research Institute, Kansas City, Mo., presented a gift of 5-deazariboflavin; 5-deazaFMN was prepared by the method of Flexser and Farkas (1952) from 5-deazarbf and was purified by DEAE-cellulose column chromatography. Lumiflavin was previously synthesized using the method of Guzzo and Tollin (1963). Flavin adenine dinucleotide (FAD) was obtained from Sigma Chemical Co., St. Louis, Missouri. Riboflavin sulfate (Rbf-SO₄) was prepared by the method of Yagi (1971) and purified by repeated column chromatography using DEAE-cellulose. All derivatives were assessed pure by thin-layer chromatography on cellulose plates with 0.1 M aqueous K₂HPO₄ at pH 7.0 as the eluant.

Concentrations were determined spectrophotometrically using published values for the molar extinctions.

Flavoproteins

The Shethna flavoprotein was isolated from Azotobacter vine-
landii, strain Wisconsin "O", using a modified procedure (Edmondson 1970) originally developed by Hinkson and Bulen (1967).
Peptostreptococcus elsdenii flavodoxin was a gift from Dr. S. G. Mayhew, University of Michigan, Ann Arbor, Michigan. Protein concentrations were obtained spectrophotometrically using published extinction values (Table 1).

Table 1. Flavoprotein extinction coefficients.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ε</th>
<th>λ&lt;sub&gt;nm&lt;/sub&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shethna</td>
<td>FP</td>
<td>10,600</td>
<td>450</td>
</tr>
<tr>
<td>P. elsdennii</td>
<td>FP</td>
<td>10,200</td>
<td>445</td>
</tr>
</tbody>
</table>

Other Materials

Ethylenediamine tetraacetate (EDTA) was obtained from Allied Chemical, Morristown, N. J., in reagent grade. Methyl viologen was obtained from K and K Labs, Inc., Hollywood, California. Tryptophan and tyrosine derivatives were obtained from Calbiochem, Los Angeles, California. Serotonin was a gift from Dr. A. Picchioni, University of Arizona, Department of Pharmacology. Sodium dithionite (90%) was a product of Eastman Organic Chemicals, Rochester, New York; DEAE-cellulose was purchased from Calbiochem, Los Angeles, California. Distilled water was passed through a mixed bed ion exchange resin and used for making all solutions. All other materials were obtained in reagent grade.
Experimental Methods

Preparation of the Shethna Flavoprotein

*Azotobacter vinelandii*, strain Wisconsin "O", was obtained in lyophilized form from American Type Culture Collection, Washington, D.C. Cultures were started on agar slants containing 27% w/v Burks medium and 2% w/v enzyme grade sucrose (Mann). Batch quantities were grown and maintained as described by Edmondson (1970).

The procedure used for isolation of the Shethna flavoprotein was basically the same as that described by Hinkson and Bulen (1967) and Edmondson (1970) with the following changes. Cells were broken using a Beckman Ribi continuous-feed French press at 20,000 psi. This apparatus provided a higher yield of broken cells under less rigorous conditions. Cell fragments were separated by centrifugation as described by Edmondson (1970) and the supernatant retained. However, treatment with 2% protamine sulfate to precipitate nucleic acid material was found to be unnecessary. Instead, the supernatant solution was chromatographed twice over DEAE-cellulose as opposed to only once in the earlier procedure. The solution thus collected was treated with 75% (NH₄)₂SO₄ w/v and the resultant precipitate was discarded. The flavoprotein-containing supernatant was dialyzed against 0.025 M phosphate at pH 7.0. Purity was assessed spectrophotometrically by comparing the absorbance ratio of the 280 nm and 450 nm absorption peaks. A good ratio of $A_{280}/A_{450}$ was considered to be in the range 5.2-4.8/1.

The yields of pure protein were consistently higher than those obtained using the protamine sulfate treatment. Concentrations were determined
using a molar extinction of oxidized flavoprotein at 450 nm of 10,600 M$^{-1}$cm$^{-1}$. Highly concentrated solutions of flavoprotein could be made by either crystallization (Edmondson 1970) or by using a Dow Minidializer in the vacuum mode.

The preparation of the apoprotein, using 3% trichloroacetic acid and dithiothreitol, was identical to that described by Edmondson (1970). The concentration of apoprotein was determined spectrophotometrically using a molar extinction of 29,000 M$^{-1}$cm$^{-1}$ at 280 nm.

**Partially Reduced Flavin Solutions**

An anaerobic reduction apparatus (Figure 2) was used for the preparation of partially reduced flavin samples for electron spin resonance and temperature-jump experiments. The apparatus was double-armed with a 1-mm path length cell on one arm and a 10-mm path length cell on the other. The two path length cells permitted the determination of absorption spectra of concentrated, oxidized flavin using the 1-mm cell and also the weak absorption spectrum of semiquinone flavin using the 10-mm cell. Reduction was accomplished by irradiating the flavin sample, which contained a 50-molar excess of EDTA to FMN, with a 650 watt, heat-filtered, Sylvania "Sun Gun". Prior to irradiation, the sample was deoxygenated by alternate evacuation and nitrogen purging and left under a slight positive nitrogen pressure. To effect half-reduction, the sample was equally divided between the two arms. Only one arm was irradiated while the other was kept dark. Irradiation was continued until the sample showed no fluorescence under uv light, thus indicating
Figure 2. Anaerobic reduction apparatus with aqueous ESR cell.
complete reduction. The samples from the two arms were then mixed, yielding a half-reduced mixture.

For temperature-jump studies, the partially reduced solution was transferred anaerobically to the temperature-jump cell within a nitrogen-filled glovebag. The temperature-jump cell was kept under a positive nitrogen pressure with constant purging. Total flavin concentrations ranged from $5 \times 10^{-4}$ M to $5 \times 10^{-3}$ M with a 50-molar excess of EDTA and 0.1 M KNO₃ held constant. All kinetics were determined at 10°C unless otherwise stated.

For electron spin resonance measurements, the reduction apparatus was fitted with an aqueous solution ESR cell which could be made anaerobic. The half-reduced sample was forced into the ESR cell by inverting the entire apparatus within a glovebag. By momentarily opening the bottom stockcock, the positive nitrogen pressure could be made to fill the cell. Flavin concentrations were $10^{-3}$ M (total flavin).

All reductions were carried out at pH 4.5 or pH 9.0, generating upon mixing the neutral and anionic form of the semiquinone, respectively. Acetate buffer (0.05 M) was used at pH 4.5, while glycine buffer (0.05 M) was used at pH 9.0. All samples contained 0.1 M KNO₃ whether they were used for T-jump or ESR experiments.

**Isolation of a Paramagnetic Precipitate from Concentrated Half-reduced Flavin Solutions**

For samples in which the total flavin concentration was above $5 \times 10^{-3}$ M, a flocculent precipitate formed upon mixing oxidized and reduced flavin. This same behavior had been observed previously
The precipitate was green at pH 4.5 and red at pH 9.0. It was possible to collect this species as a paste by anaerobic centrifugation at 20,000 xg. The paste could then be freeze-dried anaerobically by vacuum distillation and stored indefinitely without decomposition. Even under air, decomposition was relatively slow (many hours).

**Determination of Mul Absorption Spectra**

It was of interest to characterize the freeze-dried precipitate isolated from the concentrated partially reduced FMN samples. The determination of its absorption spectrum was thus of importance. Since the sample could undergo disproportionation in aqueous solutions and was found to be insoluble in organic solvents, the spectrum of a solid sample had to be determined. Inclusion of the material in potassium bromide pellets did not yield reproducibility. However, mul spectra in mineral oil proved to be quite reproducible. The freeze-dried precipitate was ground to homogeneity into deoxygenated mineral oil using a mulite mortar and pestle. This was done within a nitrogen glovebag to prevent oxidation. Spectra were determined using anaerobic Thunberg cuvettes with the Cary 14R. Opal glass was used in both the monitoring and reference beams to negate scattering due to the sample particles. Mineral oil was used as a reference.

**Determination of Relative Spin Concentrations of Solutions of Flavin Semiquinone**

For aqueous solutions of flavin containing semiquinone, relative spin concentrations were determined as a function of pH. Samples
which were 1 x 10^{-3} M in total flavin were half-reduced at pH 4.5 and pH 9.0. The relative radical concentrations were approximated by comparing the maximum peak to peak height of the ESR absorption curves at the two pH's. Instrument parameters were held constant. The sample was over-modulated such that only a broad, unstructured resonance was observed.

**Determination of Absolute Spin Concentration per Molecule of FMN in the Paramagnetic Precipitate**

Electron spin resonance spectra were determined for the solid paramagnetic precipitate obtained from reduction of concentrated flavin solutions. Samples were ground and sealed in 2-mm cylindrical quartz tubes under a nitrogen atmosphere. Spin concentrations were compared with known spin concentration standards made by grinding DPPH into dry KBr. The DPPH concentrations were determined spectrophotometrically by dissolving the standard in a measured volume of chloroform and using an extinction of 37 g^{-1} cm^{-1} at 529 nm. It was assumed that there was one spin per DPPH molecule. Total flavin concentrations were determined by dissolving the solid sample in a measured quantity of oxygenated water and determining the absorbance at 445 nm.

Because the resonances of DPPH and flavin semiquinone overlap, simultaneous determination of spectra could not be performed. Therefore extreme care was taken to keep all instrument parameters constant for both DPPH and flavin samples.
The actual integration of the absorption curves was done by a computer program using a double summation method. The program is detailed later in this section.

The number of spins per molecule of flavin was calculated by dividing the number of spins corresponding to the integrated area, as determined from the DPPH standards, by the total flavin concentration.

**Preparation of Reduced Flavoproteins**

The degree of reduction of the Shethna flavoprotein semiquinone in the pH range above 6.0 is controlled by the ionization of the fully reduced form of the flavoprotein ($pK_a = 7.0$) (Edmondson and Tollin 1971, p. 133). Even in the presence of excess dithionite, the degree of reduction will depend solely upon the pH. Therefore, by using an excess of dithionite and varying the pH in the range of 6.5 to 8.2, one can obtain equilibrium mixtures with different concentrations of semiquinone and fully reduced flavoprotein.

Samples were prepared by degassing 2 ml of flavoprotein ($3 \times 10^{-5}$ M) solution in Thunberg cuvettes and adding 1 mg of sodium dithionite from the Thunberg side arm. The pH was maintained with 0.1 M $\text{KH}_2\text{PO}_4$. Methyl viologen (10-40%) was added to increase the reduction rates (Edmondson and Tollin 1971, p. 133). Concentrations were determined spectrophotometrically at 580 nm, using an extinction of $5,300 \text{ M}^{-1}\text{cm}^{-1}$ for the semiquinone. Fully reduced flavoprotein concentrations were determined as the difference between the semiquinone concentration and the total flavoprotein concentration before reduction.
Mixtures of oxidized and half-reduced flavoprotein were prepared by adding a less than stoichiometric amount of dithionite to degassed flavoprotein-containing methyl viologen. This was done at pH 6.5 in 0.1 M KH$_2$PO$_4$ buffer.

At pH 8.2 an equilibrium could be established between all three redox states of the flavoprotein using methyl viologen and a less than stoichiometric amount of dithionite. Mixed tris-phosphate (0.05 M each) buffer was used to maintain the pH.

All partially reduced samples were transferred to the T-jump cell in a nitrogen-filled glovebag. The cell was maintained under a nitrogen atmosphere.

**Preparation of All Other Samples**

The kinetics of flavin complexation with several electron donors were determined at pH 7.0 in 0.01 M phosphate buffer. Samples were made up from fresh stock solutions.

The kinetics of binding of flavin analogs to the Shethna apoprotein were determined at pH 7.5 in mixed tris-phosphate buffer. Samples were prepared from stock solutions using an equimolar concentration of flavin and protein. For studies of FMN binding, the native holoprotein was used. All kinetics were determined at 9°C. For the binding of lumiflavin, riboflavin, and FMN, kinetics were also determined at 23°C.
Determination of Flavoprotein-binding Constants with Flavin Analogs

The association constants of flavin analogs to the Shethna apo­protein were determined at pH 7.0 in 0.025 M phosphate buffer. Fluorescence quenching titration of the flavin by apoprotein was used. Edmondson and Tollin (1971, p. 124) have detailed the procedure and calculations involved, and no modifications have been made. Gibbs free energies were calculated at 23°C for all analogs. Enthalpies and entropies of binding were also calculated for riboflavin, lumiflavin, and FMN by performing the titrations at 10°C.

Determination of Equilibrium Concentrations and Equilibrium Constants for the Principal Species Present in a Mixture of Oxidized and Reduced Flavin Mononucleotide

As pointed out by Gibson et al. (1962), the principal species present in a dilute (10^-4 M) solution of partially reduced FMN are oxidized flavin (FMN), fully reduced flavin (FMNH₂), semiquinone flavin (FMNH•), and a charge transfer complex of oxidized and reduced flavin (FMN - FMNH₂). The relevant equilibria are represented by the following equations:

\[
\begin{align*}
FMN + FMNH₂ & \rightleftharpoons K_{ct} FMN - FMNH₂ \\
FMN - FMNH₂ & \rightleftharpoons K_d 2FMNH•
\end{align*}
\]

Neglecting any minor equilibria involving radical dimerization, fully reduced flavin dimerization or odd complexes involving mixtures of oxidized, reduced, and semiquinone flavin, it is possible to ascertain the
equilibrium concentrations of the principal species spectrophotometrically. The reduction apparatus previously described was used and the calculations were as follows for a mixture of equal concentrations of oxidized and reduced flavin.

For the assumed mechanism the concentration of FMN by mass balance is

$$[\text{FMN}] = \frac{[\text{FMN}]_{\text{initial}} - [\text{FMN} - \text{FMNH}_2] - \frac{[\text{FMNH}^\cdot]}{2}}{2}$$

(1)

and similarly the concentration of FMNH$_2$ is

$$[\text{FMNH}_2] = \frac{[\text{FMNH}_2]_{\text{initial}} - [\text{FMN} - \text{FMNH}_2] - \frac{[\text{FMNH}^\cdot]}{2}}{2}$$

(2)

Spectrally, the only species absorbing in the region 300-500 nm are FMN, FMNH$_2$, and FMNH$^\cdot$. The only species absorbing beyond 900 nm is FMN-FMNH$_2$. Thus one can write

$$A_\gamma = A_\gamma^{\text{FMN}} + A_\gamma^{\text{FMNH}_2} + A_\gamma^{\text{FMNH}^\cdot}$$

$$= e_{\gamma}^{\text{FMN}} [\text{FMN}] + e_{\gamma}^{\text{FMNH}_2} [\text{FMNH}_2] + e_{\gamma}^{\text{FMNH}^\cdot} [\text{FMNH}^\cdot]$$

(3)

where $A_\gamma$ is the total absorbance and $e_\gamma$'s are extinctions in the region 300 nm $< \gamma < 500$ nm.

It is also true that:

$$A'_\gamma = A'_\gamma^{\text{FMN-FMNH}_2} = e_{\gamma}^{\text{FMN-FMNH}_2} [\text{FMN} - \text{FMNH}_2]$$

(4)

where $A'_\gamma$ is the total absorbance in the region 900 nm $< \gamma' < 1100$ nm.
Thus, substituting mass balance equations (1) and (2) into equation (3), one obtains equation (5):

\[
A_\gamma = \varepsilon_{\gamma,FMN} \frac{[FMN]}{2} \text{initial} - \frac{[FMN - FMNH_2]}{2} - \frac{[FMNH^*]}{2} + \varepsilon_{\gamma,FMNH_2} \frac{[FMNH_2]}{2} \text{initial} - \frac{[FMN - FMNH_2]}{2} - \frac{[FMNH^*]}{2} + \varepsilon_{\gamma,FMNH^*} [FMNH^*]
\]

It is also possible to substitute into equation (5) for \([FMN - FMNH_2]\) using equation (4) and obtain equation (6):

\[
A_\gamma = \varepsilon_{\gamma,FMN} \frac{[FMN]}{2} \text{initial} - \frac{A'_\gamma}{\varepsilon'_{\gamma,FMN-FMNH_2}} - \frac{[FMNH^*]}{2} + \varepsilon_{\gamma,FMNH_2} \frac{[FMNH_2]}{2} \text{initial} - \frac{A'_\gamma}{\varepsilon'_{\gamma,FMN-FMNH_2}} - \frac{[FMNH^*]}{2} + \varepsilon_{\gamma,FMNH^*} [FMNH^*]
\]

Equation (6) can be simplified and rewritten as below:

\[
A_\gamma = \frac{A_{\gamma,FMN}}{2} \text{initial} + \frac{A_{\gamma,FMNH_2}}{2} \text{initial} - \frac{A'_\gamma}{\varepsilon'_{\gamma,FMN-FMNH_2}} (\varepsilon_{\gamma,FMN} + \varepsilon_{\gamma,FMNH_2}) - \frac{[FMNH^*]}{2} (\varepsilon_{\gamma,FMN} - \varepsilon_{\gamma,FMNH_2} - 2 \varepsilon_{\gamma,FMNH^*})
\]

\(A_\gamma, A_{\gamma} \text{ (FMN) initial, } A_{\gamma} \text{ (FMNH}_2\text{) initial, and } A_{\gamma e} \text{ are experimentally determined variables. } \varepsilon_{\gamma,FMN} \text{ and } \varepsilon_{\gamma,FMNH_2} \text{ are known and } \varepsilon_{\gamma,FMNH^*} \).
has been published by Land and Swallow (1969) from pulse radiolysis experiments. Therefore, determination of the above quantities at three wavelengths (360, 445, 1100 nm) will give rise to two equations with two unknown variables, \( e_{\text{FMN-FMNH}_2} \) and \([\text{FMNH}^\cdot]\), which can be calculated by simultaneous solution. We then have all the necessary information to go back to the equations of mass balance (1) and (2) and solve for the concentrations of all species. Having the concentrations, we can solve for the equilibrium constants \( K_{ct} \) and \( K_d \), from equations (8) and (9).

\[
K_{ct} = \frac{[\text{FMN} - \text{FMNH}_2]}{[\text{FMN}] [\text{FMNH}_2]} \quad (8)
\]

\[
K_d = \frac{[\text{FMNH}^\cdot]^2}{[\text{FMN} - \text{FMNH}_2]} \quad (9)
\]

**Temperature-jump Spectrophotometer**

A joule heating temperature-jump spectrophotometer was constructed for the measurement of rapid and multiple step reaction rates. The basic design is similar to that first proposed by Eigen (1954) and is depicted in Figure 3. The working of the instrument is as follows. A Sorensen Model 230-6P-RD high voltage D.C. power supply is used to charge a high voltage 40 KV low inductance 0.1 uF capacitor (Plastic Capacitor, Inc., Chicago, Ill.). A 20-megohm power resistor is used in series between the power supply and capacitor to limit the rate of charge. The charged condenser is isolated from the sample cell and ground by connection in series with an E. G. & G. model 14B spark gap which has a maximum hold-off capability of 40 KV. The energy from the
Figure 3. Schematic diagram of the temperature-jump spectrophotometer.
capacitor is delivered through the sample cell to ground by ionizing the neon gas within the spark gap. This is accomplished by the use of a 30 KV high frequency pulse applied to the trigger electrode of the spark gap by a compatible E. G. & G. model TR60 trigger module. The trigger module is protected from the capacitor discharge by 30 KV door knob 0.50 pF capacitors. The trigger is activated by a simple shorting switch. Both the high voltage capacitor and spark gap are encased in an air-insulated plexiglass box which in turn is encased in a grounded steel chassis. The trigger module is mounted within the same steel chassis.

Joule heating is effected by passing the condenser discharge between two electrodes immersed within the sample contained in the temperature-jump cell. A conducting electrolyte (0.1 M KNO$_3$) delivers the charge uniformly across the sample to ground. A detailed treatment of the cell is presented in the next section. Treatment of relaxation kinetic theory has been presented in detail in the literature (Eigen and DeMaeyer 1963, Alberty and Hammes 1960, Castellan 1967).

The shift in equilibrium concentrations of a reacting system following joule heating is monitored spectrophotometrically. Both absorption and fluorescence detection modes can be used. In the absorption mode, monochromatic light is passed directly through the sample to a photomultiplier detection circuit. In the fluorescence mode, the sample is monochromatically irradiated at right angles to the detector. An appropriate Corning sharp cut glass filter is placed between the
sample and detector to pass the emitted fluorescence and filter out any scattered exciting light.

A General Electric type EKG Quartzline lamp (80 watts), driven by a Sorensen Q-Nobatron (QB 18-6) highly stabilized D.C. power supply, and passed through a Bausch and Lomb 250-mm grating monochromator, provided the light source.

The detector circuit consisted of a Mu-metal shielded EMI 9656-KQB super "S" - 13 quartz window photomultiplier tube driven by a Power Designs Pacific, Inc., model 2K-10 highly stabilized D.C. power supply. Both load resistance and capacitance were variable, giving a continuously variable R.C. filter with a response time from 1 usec to 100 msec. The output from the detector was fed into a Taktronix model 533A oscilloscope. Transients were recorded using a C-27 Tektronix Oscilloscope Camera with Polaroid type 107 film.

Care was taken to shield the detection circuit from the electromagnetic field accompanying the discharge. All cables were ground shielded as were all chassis. Detector leads were kept perpendicular to the discharge circuit. In addition, in order to minimize the heating time, low impedance cable was used throughout and all connections were silver soldered. The length of all cables was also minimized.

Temperature-jump Cell

Of extreme importance to the operation of a temperature-jump spectrophotometer is the design of the heating cell. In order to serve our specific needs we required a cell that would allow monitoring of both absorption and fluorescence changes, utilize a small sample volume,
and provide a wide range of accessible relaxation times. The cell which was used in these studies is a conglomerate based upon our own experience with Eigen-type cells (1954) and a cell designed by Czerlinski (1962). Detailed drawings of the cell and its specialized windows are given in Figure 4.

The materials used include a brass cap heat sink, type 316 non-magnetic stainless steel electrodes, a lucite body, and dynasil non-fluorescent quartz windows. All machining of parts, with the exception of the quartz windows, was done in the Department of Chemistry shop to exacting specifications. The quartz windows were custom-made to our specifications by M. P. Wirick Optical, Los Angeles, California.

The cell is mounted in a thermostated brass sleeve which is encased in an electrically and thermally insulated plastic box. The brass sleeve is wrapped with copper tubing through which alcohol is pumped to provide temperature control. The temperature of the coolant is maintained by a Forma Scientific refrigerated constant temperature bath. The top electrode of the cell is threaded into the brass cap of the cell which is in thermal and electrical contact with the cell-holding brass sleeve. The brass sleeve is at ground potential and therefore completes the discharge circuit.

The bottom electrode is threaded into a brass plug which in turn fits into another brass receptacle located at the bottom of the outer plastic box. This electrode is also thermostated to minimize convection within the cell due to thermal gradients. The bottom
Figure 4. Full scale drawing of the T-jump cell with lens dimensions.
electrode is separated from the high voltage capacitor by the above-mentioned spark-gap.

A prerequisite to successful operation of a temperature-jump apparatus is to assure that the capacitor discharge is conducted through the sample rather than arcing between the electrodes. In order to prevent such arcing, the interior of the cell was carefully polished with a slurry of fine alumina. In addition, the windows were slightly recessed so that no sharp edges protruded into the heated volume of the cell. Moreover, the flat surfaces of the electrodes were polished to a mirror finish with 0.3 micron Linde "A" alumina. In the present design, the edges of the electrodes are hidden from one another by the plexiglass body of the cell and thus need not be rounded as in Eigen-type cells.

The cell which we have used in these studies has several extremely important advantages over conventional cells. It only requires slightly more than one ml of solution to fill it (as opposed to 7 to 10 ml in other designs). The use of conical lenses as windows greatly enhances its optical sensitivity in both the absorption and fluorescence detection modes. Indeed, the conical lenses proved to be an absolute necessity for the fluorescence detection of kinetic transients in the present work. The lens specifications, as given in Figure 4, are the same as that proposed by Czerlinski (1962).

Inasmuch as virtually all of the sample is held between the electrodes and heated, there is no reservoir of cooler sample to mix with the heated liquid following a temperature jump. This extends the
upper limit of accessible relaxation times to more than thirty seconds before appreciable cooling occurs. As will be seen, this capability has been quite important in these studies.

The exponential heating time of the cell was determined using a buffer-indicator sample which equilibrates during the temperature rise. For this purpose 0.01 M phosphate buffer containing $2 \times 10^{-5}$ M phenol red at pH 7.2 was used. A plot of $\ln \Delta A$ vs. time yielded a straight line with a slope equal to the reciprocal exponential heating time (Figure 5). The heating time was found to be 11 $\mu$sec and is independent of the applied voltage.

The temperature rise of the cell as a function of applied voltage was also calculated using this phosphate-phenol red buffer system. This was done using the absorbance changes of the buffer system following heating, in conjunction with the known enthalpy and the Gibbs-Helmholz equation:

$$\ln \frac{K_1}{K_2} = \frac{\Delta H(T_2 - T_1)}{RT_2T_1}$$

For most experiments, a potential of 20 KV was used. This gave a 7°C rise. In addition, most kinetic constants were determined at 9°C by thermostating the cell at 2°C. The use of this temperature range minimized sample cavitation due to the thermal expansion of water.

**Stopped-flow Measurements**

For the determination of the binding kinetics of several flavin analogs to the Shethna apoprotein, a Gibson-Durrum stopped-flow
Figure 5. Plot for the determination of the exponential heating time of the temperature-jump apparatus.

Phenol red (2 x 10^{-5} M) in phosphate buffer (0.05 M) at pH 7.2.
spectrophotometer was used. This instrument was modified for fluorescence detection by replacing the normal observation cell with a fluorescence cell. The light source which is normally used for absorption detection served as an excitation beam and the fluorescent emission was passed through an appropriate cut-off filter to a Hamamatsu TA0285 photomultiplier tube. The photomultiplier tube and filter were mounted at a right angle to the excitation beam and were less than a centimeter away from the sample cell window. Experimentally, fluorescence quenching was observed resulting from the binding of flavin to the apoprotein.

In all stopped-flow experiments, flavin and apoprotein were kept in equal initial concentrations. This greatly simplifies the treatment of data since under these conditions a plot of the reciprocal concentration of free flavin vs. time should be linear for a second-order process. Inasmuch as fluorescence intensity is directly proportional to concentration, a plot of the reciprocal of the fluorescence intensity vs. time yields the second order rate constant as seen from the following equation:

\[
\frac{1}{F} = k t + \frac{1}{F_0}
\]

The fluorescence intensity was corrected for scattered light by using appropriate blanks of apoprotein and buffer.

**Flash Photolysis Experiments**

Flash experiments were performed on a flash photolysis spectrophotometer constructed by myself and previously described by Vaish and Tollin (1970). For all experiments, samples were extensively
degassed by six cycles of freeze-thawing under a vacuum of $10^{-6}$ torr. Five and ten cm cylindrical quartz Cary cells were used.

Flash-produced transients were displayed and photographed on a Tektronix 533A oscilloscope. Transient absorption spectra were recorded by holding the transmitted monitoring light intensity constant at all wavelengths and plotting the maximum transient concentration (peak height) as a function of monitoring wavelength. Correction was not made for phototube sensitivity. Extinctions were not calculated and only relative absorption was determined.

**Spectral Measurements**

Absorption spectra were determined using a Coleman-Hitachi model 124 double-beam spectrophotometer or a Cary 14R recording spectrophotometer. For concentration determinations, a Gilford model 240 single-beam spectrophotometer was used. A Cary model 60 spectropolarimeter with a model 6001 CD attachment was used for circular dichroism spectra.

Quartz cells from Precision Cells, Inc., Hicksville, New York, were used for aerobic spectra. Both pyrex and quartz Thunberg cuvettes obtained from Precision Cells, Inc., were used for anaerobic spectra.

Fluorescence measurements were performed on high sensitivity fluorimeter constructed by Dr. J. A. D'Anna, Jr., in this laboratory (D'Anna and Tollin 1971), and modified to use a Bausch and Lomb high intensity monochrometer for the selection of the excitation wavelength.

A Varian V-4501 electron paramagnetic resonance spectrometer was used for ESR measurements. A Varian anaerobic aqueous sample cell
was fitted with a double arm irradiation cell (Figure 2) for the determination of ESR spectra of half-reduced flavin solutions. The cell was filled with a slightly positive pressure of nitrogen.

Electron spin resonance measurements of solid samples were performed in quartz tubes that were sealed by torch under a nitrogen atmosphere.

Spectra for determination of redox potentials were measured in a 1-cm rectangular pyrex cell which contained three ground-glass side arms for the insertion of salt bridges and working and/or reference electrodes. Both the Cary 14R and Coleman-Hitachi spectrophotometers were used in these measurements. The reduction cell was stirred by a magnetic bar to maintain a uniform potential. All spectra were run at ambient temperatures (28° ± 2°C).

**Reduction Potential Measurements**

Reduction potentials were determined for the Shethna flavoprotein by coulometric reduction and also by potentiometric titration with sodium dithionite. Coulometric reductions were performed with the aid of Dr. George Wilson of the Department of Chemistry using a small-volume "Coulometric Redoxstat" which he designed (Swartz and Wilson 1971). In this method, the reductant is coulometrically generated within the sample by applying current to a generating electrode pair. The potential of the sample is sensed by a platinum indicating electrode and compared with a standard Ag-AgCl electrode. Any desired potential can be chosen and the degree of reduction monitored spectrophotometrically. In the present work, methyl viologen was used as the
redox buffer and primary reductant. This method is particularly applicable to slowly established equilibria inasmuch as any given potential within the sample cell can be maintained indefinitely.

Potentiometric titrations were performed in the anaerobic three-arm cuvette described above. Reductant was added from a micro-syringe placed in one arm, an indicating platinum electrode occupied another arm, and an Ag-AgCl reference electrode was placed in the third arm. Methyl viologen (5 mol percent) was added to mediate the reduction of the flavoprotein by dithionite. The cell potential relative to the Ag-AgCl reference electrode (+0.200 V) was monitored using a high impedance digital voltmeter and corrected to the standard hydrogen electrode potential. The degree of reduction of the flavoprotein was monitored spectrophotometrically using the Coleman-Hitachi spectrophotometer. All potential measurements were performed at pH 8.2 in 0.1 M tris buffer. The degree of reduction was calculated from the appearance and subsequent disappearance of 580 nm absorbance of the Shethna flavoprotein semiquinone. The one- and two-electron potentials were found to be sufficiently separated to permit this procedure. The concentrations of the semiquinone were calculated using a molar absorptivity of 5,600 M\(^{-1}\)cm\(^{-1}\) at 580 nm. The corresponding concentrations of oxidized flavoprotein were calculated using the difference between the initial oxidized concentration and the semiquinone concentration. The concentrations of the fully reduced flavoprotein were calculated using the difference between the maximum semiquinone concentration and the concentration of semiquinone upon further reduction. Correction was made
for the absorption due to reduced methyl viologen at each potential. The standard reduction potentials were then calculated, using the Nernst equation, from a plot of \( \log \frac{\text{red}}{\text{ox}} \) vs. the cell potential, in which the slope was equal to the constant factor \( \frac{RT}{nF} \) and the intercept equal to \( E^0 \).

**Program to Double Integrate ESR Absorption Curves**

A Fortran II program using progressing sums was written to carry out the double integration of ESR absorption curves. The method and mathematics are as follows. The abscissa of the first derivative spectra was divided into \( N \) equal division of constant width \( \Delta \). The ordinate, \( H(I) \), was measured in the center of each \( \Delta \) division as the height of the absorption curve. The height of the integrated absorption curve at the end of the \( I^{th} \) division, \( \text{Aabsop}(I) \), can then be calculated from the following equation.

\[
\text{Aabsop}(I) = \Delta \sum_{N=1}^{I} H(I)
\]

(12)
The integrated absorption curve can be approximated by a polygon with \( N \) units and the \textit{Area} beneath this curve is given by

\[
\text{Area} = \frac{1}{2} \Delta^2 \sum_{i=1}^{N} (2N - 2i + 1) H(i)
\]  

(12)

This \textit{Area} is a good approximation to the actual area only when there is no asymmetry caused by base line drift as shown below.

Such a base line drift error can be corrected for by measuring the ordinates, \( H(i) \), from the apparent base line obtained by joining the ends of the integrated spectrum by a straight line. The area in the pie-shaped slice between the original base line and the apparent base line is then calculated as \textit{Error}.

\[
\text{Error} = \frac{1}{2} \Delta^2 \sum_{i=1}^{N} H(i)
\]  

(14)

The \textit{Error} term is depicted below.
The corrected area, Coarea, is obtained by subtracting Error from Area and is depicted below.

Any errors due to the presence of underlying broad absorptions or the mixing of absorption and dispersion signals are not accounted for in this correction.

It is advisable to check the height of the past point on the integrated absorption curve (Sum), which should be close to zero.

\[ \text{Sum} = \sum_{I=1}^{N} H(I) \]  \hspace{1cm} (15)

Sum gives an indication of the symmetry of the integrated absorption curve. The derivation of this approach to area calculation has been given by Ayscough (1967).
Analog Computer Resolution of Kinetics

An analog computer was used to separate the relaxation components for multiple step reactions, whose relaxation times were not sufficiently different to allow graphical resolution. The computer was programmed to synthesize up to three exponential curves, for which the time constants and amplitudes \((A = e^{-kt})\) could be varied independently by means of six-digital potentiometers. The circuitry was such that exponential curves could be computed one at a time, or the sums of two or three exponential curves computed simultaneously. The resultant computations were displayed on a Tektronix 533A oscilloscope. The experimentally determined relaxation spectrum was superimposed on the oscilloscope screen and was fitted with a computer derived trace. The fit was accomplished by varying the time constant and amplitude of the individual computer synthesized exponential traces and summing these. A minimum of exponentials was used to achieve a reasonable fit.

The computed exponentials, whose sum gave the best experimental fit, were displayed one at a time and photographed. Semilog plots of signal height (arbitrary units) vs. time were made, and the individual relaxation times calculated from the slopes.
RESULTS AND DISCUSSION

Kinetics of Complex Formation of Flavins with Aromatic Compounds

It is well established that flavins will form complexes with many aromatic compounds (Tollin 1968). Of particular interest is the complexation with tryptophan and tyrosine (Isenberg and Szent-Gyorgi 1958) since such complex formation may be responsible in part for binding of flavins in flavoproteins (Tollin 1968, Draper and Ingraham 1970). Also of interest is the binding of other indoles to flavins, since they may serve as models for this process. In view of this, it was of interest to study the kinetics of complexation of FMN with tryptophan, tyrosine, serotonin, 2,3-naphthalenediol, quinoxaline, and 3-methyl indole. However, in all experiments, even at concentrations of $10^{-5}$ molar, the kinetics were faster than the heating time of the temperature-jump apparatus. Therefore, it was only possible to obtain lower limits on the rate constants using published equilibrium data (Table 2). The calculated rate constants are more than two orders of magnitude larger than those observed for flavin binding to apoproteins (Edmondson and Tollin 1971, p. 124, and this work), and it thus appears that even though flavin complexation with tryptophan and tyrosine may be important in the flavin-protein interaction, it certainly is not a rate-determining interaction for the binding process (see section on binding of flavin analogs to apoprotein). It appears that the kinetics may be diffusion-controlled and, if so, little can be learned from
Table 2. Minimum rate constants for FMN complexation.

<table>
<thead>
<tr>
<th>Aromatic donor</th>
<th>Bimolecular rate constant (M(^{-1})sec(^{-1}))</th>
<th>Monomolecular rate constant (sec(^{-1}))</th>
<th>Association constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>(&gt; 1 \times 10^7)</td>
<td>(&gt; 1 \times 10^5)</td>
<td>90(^a)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>(&gt; 1 \times 10^7)</td>
<td>(&gt; 1 \times 10^5)</td>
<td>66(^a)</td>
</tr>
<tr>
<td>Serotonin</td>
<td>(&gt; 4 \times 10^7)</td>
<td>(&gt; 1 \times 10^5)</td>
<td>400(^a)</td>
</tr>
<tr>
<td>3-Methyl-indole</td>
<td>(&gt; 2 \times 10^7)</td>
<td>(&gt; 1 \times 10^5)</td>
<td>50(^b)</td>
</tr>
<tr>
<td>Quinoxaline</td>
<td>(&gt; 1 \times 10^7)</td>
<td>(&gt; 1 \times 10^5)</td>
<td>100(^c)</td>
</tr>
<tr>
<td>2,3-naphthalenediol</td>
<td>(&gt; 2 \times 10^7)</td>
<td>(&gt; 1 \times 10^5)</td>
<td>240(^d)</td>
</tr>
</tbody>
</table>

b. Tollin (1968)
c. Estimated.
d. Fleischman and Tollin (1965).
Kinetic measurements about the intricacies of charge transfer complexa-
tion of indoles with flavins.

**Kinetics and Mechanisms of Reactions in Partially Reduced FMN Solutions**

Gibson et al. (1962) have shown that the principal species present in a dilute (10^{-3} M) partially reduced FMN solution are oxidized flavin (F), fully reduced flavin (FH_2), half-reduced semiquinone (FH^*), and a complex between oxidized and reduced flavins (F-FH_2). The following scheme was suggested to be operative:

\[
\begin{align*}
F + FH_2 & \overset{k_1}{\rightleftharpoons} F - FH_2 & \overset{k_3}{\rightleftharpoons} 2FH^* \\
\end{align*}
\]

where the complex (F-FH_2) is a precursor to the semiquinone. Swinehart (1965), using the temperature-jump method, calculated the rate constants for such a scheme. However, his estimate of \(k_4 (6 \times 10^6 \text{ M}^{-1}\text{ sec}^{-1})\) was three orders of magnitude less than that obtained using flash photolysis (3 \times 10^9 \text{ M}^{-1}\text{ sec}^{-1}) to study the disproportionation of flavin radicals (Vaish and Tollin 1970). This discrepancy could be due to several factors which will be subsequently discussed.

Flash photolysis and temperature-jump experiments were performed at different pH's. In addition, assuming the above scheme, the equilibrium constants for the two steps were calculated.

**Equilibrium Constants**

The two equilibrium constants for the reactions described below
\[
F + FH_2 \xrightleftharpoons[k_2]{k_1} F - FH_2
\]  
(16)

\[
F - FH_2 \xrightleftharpoons[k_4]{k_3} 2FH^-
\]  
(17)

are \(K_{12}\) and \(K_{34}\) and are defined by equations (18) and (19).

\[
K_{12} = \frac{[F - FH_2]}{2F [FH_2]}
\]  
(18)

\[
K_{34} = \frac{[FH^-]^2}{[F - FH_2]}
\]  
(19)

These constants were determined as described in the Experimental section at both pH 9.0 and pH 4.5 and are presented in Table 3.

Table 3. Equilibrium constants for partially reduced FMN solutions.

<table>
<thead>
<tr>
<th>pH</th>
<th>(K_{12} \times 10^2) (M(^{-1}))</th>
<th>(K_{34} \times 10^{-5}) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>1.9 ± 0.2</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>9.0</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

In all determinations, the concentrations of oxidized and fully reduced flavin were kept equal. This minimized dimerization of the fully reduced flavin, which Gibson et al. (1962) consider to be negligible under these conditions.
At a given pH, the concentration of the semiquinone as a function of the concentration of oxidized and fully reduced flavin is as shown in equation (20).

$$\left[ \text{FH}^- \right]_{\text{pH}} = \sqrt{K_{12} K_{34} \left[ \text{F} \right] \left[ \text{FH}_2 \right]_{\text{pH}}}$$  \hspace{1cm} (20)

Therefore, the ratio of semiquinone concentration at pH 4.5 to that at pH 9.0, when F and FH$_2$ are equal at both pH's, is given by equation (21).

$$\frac{\left[ \text{FH}^- \right]_{4.5}}{\left[ \text{FH}^- \right]_{9.0}} = \frac{\sqrt{K_{12} K_{34} \text{pH 4.5}}}{\sqrt{K_{12} K_{34} \text{pH 9.0}}}$$  \hspace{1cm} (21)

Using the calculated equilibrium constants in Table 3, the ratio of $\frac{[\text{FH}^-]_{4.5}}{[\text{FH}^-]_{9.0}}$ is 2.7 (± 0.1). In order to test this, solutions of equal concentrations of F and FH$_2$ at the two pH's were prepared and their ESR spectra recorded. Inasmuch as the spectral line width is approximately independent of pH, the spin concentrations can be taken as proportional to the peak to peak amplitude. The spectra of equal concentrations samples at pH 4.5 and pH 9.0 are shown in Figure 6. The ratio of the peak to peak height is 2.7/1±0.1). Therefore the ESR ratio is identical with that obtained from the equilibrium constants. The equilibrium data show that the neutral flavin complex (F-FH$_2$) is about three times less stable than the anion complex (F-FH$^-$). This same conclusion is implied by the rates of radical disproportionation in flash photolysis experiments at pH 5.0 and 9.0 (Vaish and Tollin 1971). This may have biological significance inasmuch as FH$^-$ is
Figure 6. ESR spectra of half-reduced aqueous flavin solutions.

Smaller spectrum ($10^{-3}$ M FMN, 0.1 M glycine buffer, pH 9.0).

Larger spectrum ($10^{-3}$ M FMN, 0.1 M acetate buffer, pH 4.5).
probably the principal form of fully reduced flavin at physiological pH. It should be noted that the equilibrium constants determined at pH 4.5 ($K_{12} = 1.9 \times 10^2 \text{ M}^{-1}$, $K_{34} = 8.0 \times 10^{-5} \text{ M}$) are in fair agreement with the constants determined by Michaelis (1938) for riboflavin at pH 5.0 ($K_{ct} = 6.0 \times 10^2 \text{ M}^{-1}$, $K_d = 10^{-4} \text{ M}$) but differ by an order of magnitude from those estimated by Gibson et al. (1962) of $K_{ct} = 2.0 \times 10^3$, $K_d = 10^{-6} \text{ M}$ for FMN at pH 6.5. The equilibrium constants at pH 9.0 are the first such determined for the anion form of the flavin semiquinone.

**Temperature-jump Kinetics**

For the reaction sequence given by equations (16) and (17), three possible situations can occur: (16) is established rapidly compared to (17), (17) is established more rapidly than (16), or both are established in the same time range. The last possibility can be eliminated by studying the temperature-jump relaxation behavior at pH 4.5 as a function of wavelength. In the region 480 nm to 620 nm, there is a fast ($<10 \mu\text{sec}$) optical density increase followed by a slower optical density increase ($>100 \mu\text{sec}$) (Figure 7). At wavelengths beyond 680 nm (where only the $F$-$FH_2$ complex absorbs), one can only observe a fast ($<10 \mu\text{sec}$) transient corresponding to an optical density decrease (the sensitivity of the apparatus at this wavelength is much less than at the lower wavelengths). The direction of the optical density changes are the same as has been observed by studying the temperature dependence of the spectrum of partially reduced FMN solutions (Beinert 1956). From
Figure 7. Plot of log $\Delta A$ vs. time for the determination of the relaxation time for a half-reduced FMN solution (experimental trace superimposed).

Transient was recorded at 580 nm.
these observations one can say that one of the above two equilibria must be established more rapidly than the other.

An analysis of the two possible situations leads to two sets of relaxation equations with different concentration dependencies. For the case in which (16) is faster than (17), equations (22) and (23) characterize the two relaxation times,

\[ \frac{1}{\tau_1} = k_1[F + FH_2] + k_2 \quad \text{fast} \]  

\[ \frac{1}{\tau_2} = \frac{k_3 K_{12} [F + FH_2]}{1 + K_{12} [F + FH_2]} + 4k_4[FH^+] \quad \text{slow} \]  

If (17) is fast compared with (16), equations (24) and (25) hold:

\[ \frac{1}{\tau_2} = k_3 + 4k_4[FH^+] \quad \text{fast} \]  

\[ \frac{1}{\tau_2} = \frac{4k_2[FH^+]}{K_{34} + 4[FH^+]} + k_1[F + FH_2] \quad \text{slow} \]  

By considering the concentrations used in the experiments and the magnitude of the equilibrium constants \( K_{12} \) and \( K_{34} \), the above expressions for \( \frac{1}{\tau_2} \) can be simplified as follows:

\[ \frac{1}{\tau_2} = 4k_4[FH^+] + k_3 \quad (26) \]  

or

\[ \frac{1}{\tau_2} = k_1[F + FH_2] + k_2 \quad (27) \]
Therefore a plot of \( \frac{1}{\tau_2} \) vs. either \( 4[\text{FH}'] \) or \( [\text{F} + \text{FH}_2] \) should be linear depending upon which mechanism to correct. The concentration of \([\text{FH}']\) can be evaluated at 580 nm while the sum of concentrations (F and \( \text{FH}_2 \)) must be calculated from the equilibrium constants using the initial concentrations. It was found that a plot of \( \frac{1}{\tau_2} \) vs. \( 4 \times (A_{580}) \) was linear (Figure 8). However, due to the small concentration range that is experimentally accessible, a plot of \( \frac{1}{\tau_2} \) vs. \( [\text{F} + \text{FH}_2] \) also appears linear. Therefore, in order to calculate the kinetic constants, an assumption must be made as to which of the two reactions (either complex or radical formation) is faster than the other. Because the rate of flavin complexation is fast (previous section) and complexation does not involve a chemical change while radical formation does, it is probably reasonable to assume that the slower step is the production of radical (equation 17). This assumption is further supported by the fact that radical formation would involve the generation of a thermodynamically unstable species, which could tend to slow down the rate of this reaction. On the basis of this assumption, the rate constant \( k_4 \) was calculated from the slope of the plot in Figure 8, using a molar extinction of 4,700 M\(^{-1}\)cm\(^{-1}\) (Land and Swallow 1969). Using the previously calculated equilibrium constant, \( K_{34} = k_3/k_4 \), the rate constant \( k_3 \) can be calculated. We can also obtain lower limits for the rate constants \( k_1 \) and \( k_2 \) from the heating time of the instrument and the previously calculated equilibrium constant \( (K_{12}) \), since \( \frac{1}{\tau_1} = k_1[\text{F} + \text{FH}_2] + k_2 \) and \( K_{12} = k_1/k_2 \). The calculated constants are given in Table 4 together with the values obtained by Swinehart (1965) in similar
Figure 8. Plot of $1/\tau_2$ vs. $4[A_{580}]$ for the calculation of the kinetic constants for half-reduced FMN solutions at pH 4.5 (10°C).
Table 4. Rate constants for the equilibria in partially reduced FMN solution (pH 4.5) at 10°C.

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>This study</th>
<th>Swinehart (1965)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_4$</td>
<td>$1.0 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$</td>
<td>$6.0 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$</td>
</tr>
<tr>
<td>$k_3$</td>
<td>$8.3 \times 10^2 \text{ sec}^{-1}$</td>
<td>$5.0 \times 10^3 \text{ sec}^{-1}$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$&gt; 1 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$</td>
<td>$&gt; 10^9 \text{ M}^{-1}\text{sec}$</td>
</tr>
<tr>
<td>$k_1$</td>
<td>$&gt; 10^6 \text{ sec}^{-1}$</td>
<td>$&gt; 10^6 \text{ sec}^{-1}$</td>
</tr>
</tbody>
</table>
experiments. The agreement between the two sets of experiments is fair and within an order of magnitude of one another. Agreement between the two experiments would be better if Swinehart's data were recalculated using a larger value for the extinction of the flavin semiquinone \([E = \text{4700 M}^{-1}\text{cm}^{-1}] (\text{Land and Swallow 1969})\) rather than \(\text{3050 M}^{-1}\text{cm}^{-1}\) as he used]. This would yield values of \(k_4 = 9.4 \times 10^6 \text{M}^{-1}\text{sec}^{-1}\) and \(k_3 = 1.8 \times 10^3 \text{sec}^{-1}\) which agree more closely with the constants calculated in the present study. It should be noted that Swinehart used dithionite to achieve partial reduction while in the present study photoreduction with EDTA was used. Thus it appears that the method of reduction does not affect the kinetics.

From these data, it appears that the major factor controlling the rates of establishment of the two equilibria are the magnitudes of \(k_2\) and \(k_3\); i.e., the complex dissociates into \(F\) and \(FH_2\) much more rapidly than it comproportionates into radicals. It was not possible to determine the kinetics at pH 9.0 since the anion semiquinone absorption is obscured by the oxidized flavin absorption.

It should be noted that the above mechanisms assume that the complex \([F - FH_2]\) is the precursor of the semiquinone. However, there is another mechanism that also leads to a linear dependence of \(\frac{1}{\tau_2}\) on \([FH^\cdot]\):

\[
F + FH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} F - FH_2
\]  

(28)

\[
F + FH_2 \overset{k_5}{\underset{k_6}{\rightleftharpoons}} 2FH^\cdot
\]  

(29)
If we assume that the first equilibrium (28) is established more rapidly than is the second equilibrium (29), we can obtain expressions for \( \frac{1}{\tau_1} \) and \( \frac{1}{\tau_2} \) as given in equations (30) and (31).

\[
\frac{1}{\tau_1} = k_1 [F + FH_2] + k_2 \quad (30)
\]

\[
\frac{1}{\tau_2} = \frac{k_5 k_{12} [F + FH_2]}{1 + K_{12} [F + FH_2]} + 4k_6 [FH^+] \quad (31)
\]

From our equilibrium data it is possible to calculate the equilibrium constant \( K_{56} \) from equation (32):

\[
K_{56} = \frac{k_5}{k_6} = \frac{[FH^+]^2}{[F][FH_2]} \quad (32)
\]

The rate constant \( k_6 \) as calculated from the plot of \( \frac{1}{\tau_2} \) vs. \( 4[FH^+] \) is identical to \( k_4 (1.0 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}) \). From the value of \( K_{56} \) (1.5 x 10\(^{-2}\)), \( k_5 \) is found to be 1.5 x 10\(^5 \) M\(^{-1}\)sec\(^{-1}\). The rate constants \( k_1 \) and \( k_2 \) are the same as before.

The question of the actual precursor of the semiquinone, the dimer complexes \([F - FH_2]\) or the free flavin forms \((F \text{ and } FH_2)\), cannot be resolved kinetically, as both mechanisms will show the same concentration behavior. However, the results of Gibson et al. (1962) and of Fox and Tollin (1966) indicate that the dimer is a precursor to the semiquinone and that the first mechanism proposed is probably correct.
Flash Photolysis

Regardless of which of the above mechanisms is correct, the rate constants \( k_4 \) or \( k_5 \) should agree with rate constant \( k_d \) for disappearance of flavin semiquinone obtained from flash photolysis experiments. Previous work by Vaish and Tollin (1971) determined the rate constant \( k_d \) at pH 5.0 to be \( 2.6 \times 10^9 \text{ M}^{-1}\text{sec}^{-1} \). This is two orders of magnitude greater than that derived from the temperature-jump experiments. It should be noted that the temperature-jump rate constant depends upon an accurate estimation of the extinction coefficient for the free radical. However, to bring the rate constant up to the value obtained by flash photolysis, the extinction coefficient would have to be two hundred times greater than that used in the calculation (i.e., \( 10^6 \text{ M}^{-1}\text{cm}^{-1} \)), which hardly seems possible. Published extinctions for the FMN semiquinone range from 700 \( \text{M}^{-1}\text{cm}^{-1} \) (Holmstrom 1962) to 13,600 \( \text{M}^{-1}\text{cm}^{-1} \) (Gibson et al. 1962). The value of 4,700 \( \text{M}^{-1}\text{cm}^{-1} \) determined by Land and Swallow (1969) with pulse radiolysis is probably the most accurate. Therefore the discrepancy in rate constants must lie somewhere else.

In flash photolysis experiments the total flavin concentration is generally about \( 5 \times 10^{-6} \text{ M} \), and all the flavin is initially in the oxidized state. In the present temperature-jump studies, the total flavin concentration was \( >10^{-3} \text{ M} \) and equal quantities of oxidized and reduced flavin were present. Therefore, flash photolysis experiments were performed on flavin solutions that more closely paralleled those used for the temperature-jump experiments. That is, samples were made...
as concentrated in flavin as possible (5 x 10^{-4} M), the buffer and EDTA concentrations were identical to those used in the temperature-jump studies, and the sample was half-reduced initially. Higher concentrations could not be used because of the high absorbance of these solutions. Figure 9 shows a plot of 1/ΔA vs. time obtained under these conditions from which the rate constant for the disappearance of semiquinone can be calculated. Again k_d was found to be 3 x 10^9 M\(^{-1}\)sec\(^{-1}\) and good second order behavior was followed. It should be noted, however, that the sample concentration used was still only 10% or less of that used in the temperature-jump experiments.

Resolution of the Difference Between Flash Photolysis and Temperature-jump Kinetics

It was noted that when the concentration of a half-reduced FMN solution approached 10^{-2} M, a flocculent precipitate was formed. This precipitate was green at pH 4.5 and red at pH 9.0, suggesting that it might be a form of the flavin semiquinone (Beinert 1956). The precipitate was collected and characterized as detailed in the Experimental section with the following results. The mul spectra of samples obtained at pH 4.5 and pH 9.0 are shown in Figures 10 and 11, respectively. It is instructive to compare these spectra with the spectra obtained for partially reduced flavin solutions (Figures 12, 13, and 14). Examination of Figures 10 and 11 shows that at both pH's there is a broad red absorption that is similar to the broad absorption seen in solutions (Figure 14) of partially reduced flavins and is attributed to a charge-transfer complex of oxidized and reduced flavin (Gibson et al.
Figure 9. Plot of $1/\Delta A$ vs. time for the determination at the rate of radical disproportionation by means of flash photolysis at pH 4.5.

Total FMN ($5 \times 10^{-4}$ M); Total FMNH$_2$ ($5 \times 10^{-4}$ M); Acetate buffer (0.1 M).
Figure 10. M1 absorption spectrum of a paramagnetic precipitate isolated from half-reduced FMN solutions (≈10^{-2} M) at pH 4.5.
Figure 11. Mul absorption spectrum of a paramagnetic precipitate isolated from half-reduced FMN solutions ($\sim 10^{-2}$ M) at pH 9.0.
Figure 12. Absorption spectra of oxidized, half-reduced and fully reduced FMN at pH 4.5.

8 x 10^{-5} M FMN; 0.1 M acetate buffer.

- --- oxidized
- ---- half-reduced
- ------ fully reduced
Figure 13. Absorption spectra of oxidized, half-reduced, and fully reduced FMN at pH 9.0.

$9 \times 10^{-5}$ M FMN; 0.1 M glycine buffer.
Figure 14. Absorption spectra of half-reduced FMN solutions (10^{-3} M).

pH 4.5 (———)

pH 9.0 (-----)
1962) or to a radical dimer of the semiquinone (Beinert 1960). In addition, at pH 4.5 there is a shoulder around 600 nm that is absent at pH 9.0. This could be due to the absorption of the neutral semiquinone which absorbs strongly at 580 nm in solution at pH 4.5 but has no 580 nm absorption in basic solutions. There are also peaks around 450 nm and 380 nm which are characteristic of a mixture of both oxidized and fully reduced flavin (cf the enhancement of the 380 nm absorption in Figures 12 and 13).

The precipitate was also found to be paramagnetic and the percent spin per molecule of FMN was calculated as previously described. The ESR first derivative and integrated spectra are shown in Figures 15 through 18. It was found that at pH 4.5 and pH 9.0 the precipitate averaged 50.5% and 33.0% (four determinations each) spins per mole of FMN, respectively. Note that, as in solution (see above), the low pH system has the higher spin concentration.

The above experiments lead to the conclusion that the precipitate is most likely a complex of oxidized and reduced FMN that is in equilibrium with a semiquinone pair. Thus, it may be represented by the following equilibrium:

\[
(F - FH_2) \rightleftharpoons (FH\cdot - FH\cdot)
\]

It is noteworthy that there is narrowing of the peak to peak line width (\(\Delta H\)) in going from the pH 4.5 complex to the pH 9.0 complex. Such a line narrowing may result from a faster electron exchange rate between the two flavins in the pH 9.0 complex. At pH 9.0 the fully reduced and
Figure 15. First derivative ESR absorption spectrum of the FMN paramagnetic precipitate (pH 4.5).
Figure 16. Integrated ESR absorption spectrum of the FMN paramagnetic precipitate (pH 4.5).
Figure 17. First derivative ESR absorption spectrum of the FMN paramagnetic precipitate (pH 9.0).
Figure 18. Integrated ESR absorption spectrum of the FMN paramagnetic precipitate (pH 9.0).
radical forms of FMN are largely in the anion state due to the loss of a proton. These would be expected to pick up a metal cation (K⁺) upon precipitation. This could act to mediate electron exchange and thus account for the narrowing of the ESR signal. Substitution of a metal cation for a proton might also change the spin distribution with a possible narrowing of ΔH. Furthermore, it is reasonable to expect that the spin dipole-dipole interaction would be different in complexes of different states of protonation.

To further document this model, the stoichiometry of the solids was determined by anaerobically dissolving a measured weight of the complex in degassed buffer and recording the spectra. In sufficiently dilute solution (< 10⁻⁴ M) the above species should dissociate to give an equimolar mixture of oxidized and reduced FMN. Other complexes that can be imagined as being present in partially reduced flavin solution would yield odd ratios of oxidized to reduced flavin upon dilution. The possible models and their dissociation behavior are represented by the following equations (33-38):

\[
\begin{align*}
[F - FH_2] & \rightleftharpoons [FH^+ - FH\cdot] \rightleftharpoons F + FH_2 \\
[F - 2FH_2] & \rightleftharpoons F + 2FH_2 \\
[F - FH\cdot] & \rightleftharpoons 1.5F + 0.5FH_2 \\
[FH_2 - FH\cdot] & \rightleftharpoons 1.5FH_2 + 0.5F \\
2F - FH_2 & \rightleftharpoons 2F + FH_2 \\
[FH_2 - FH_2] & \rightleftharpoons 2FH_2
\end{align*}
\]
In Table 5 the experimentally determined optical densities are shown together with the calculated optical densities for each of the models. The calculated values were determined from the total flavin concentration upon oxidation of the solution. In all the samples tried a one-to-one correspondence of oxidized and reduced FMN was found; this correspondence is consistent with the conclusions arrived at from the ESR and mul spectra experiments. It is thus possible that the species absorbing at 900 nm, which is called a charge-transfer complex by some and a radical dimer by others, is probably best characterized as a mixture of these two types.

It is now possible to account for the difference in radical disproportionation kinetics determined by flash and temperature-jump experiments. The temperature-jump experiments were performed in a concentration range that permits appreciable complex formation and dimerization of the flavin semiquinone. Therefore, an additional step must be included in the mechanism of semiquinone formation [equations (16) and (17)] to account for the dimerization process [equation (39)].

\[
F + FH_2 \overset{k_1}{\underset{k_2}{\rightleftharpoons}} F - FH_2 \overset{k_3}{\underset{k_4}{\rightleftharpoons}} [FH\cdot]_2 \overset{k_5}{\underset{k_6}{\rightleftharpoons}} 2FH\cdot 
\]

If we assume that the first two steps are fast (complexation and dimerization) compared to the third step (radical formation), this mechanism can be simplified to equation (40).

\[
[FH\cdot]_2 \overset{k_5}{\underset{k_6}{\rightleftharpoons}} 2FH\cdot 
\]
Table 5. Determination of the stoichiometry of the FMN paramagnetic complex at pH 4.5.

<table>
<thead>
<tr>
<th>Trial</th>
<th>$\gamma_{nn}$</th>
<th>Calculated absorbance</th>
<th>Experimental absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-FH₂</td>
<td>F-2FH₂</td>
<td>F-FH⁺</td>
</tr>
<tr>
<td>1</td>
<td>0.55 0.57</td>
<td>0.58 0.56</td>
<td>0.83 0.94</td>
</tr>
<tr>
<td>2</td>
<td>0.65 0.68</td>
<td>0.54 0.52</td>
<td>0.79 0.89</td>
</tr>
<tr>
<td>3</td>
<td>0.39 0.41</td>
<td>0.33 0.31</td>
<td>0.47 0.53</td>
</tr>
<tr>
<td>4</td>
<td>0.43 0.45</td>
<td>0.34 0.34</td>
<td>0.52 0.59</td>
</tr>
<tr>
<td>5</td>
<td>0.70 0.74</td>
<td>0.47 0.58</td>
<td>0.88 0.99</td>
</tr>
</tbody>
</table>
and the expression for the relaxation time would be identical with equation (26), except that $k_3$ and $k_4$ have now been replaced by $k_5$ and $k_6$. A plot of $1/\tau_2$ vs. $4[A_{580}]$ should now yield the rate constants $k_5$ and $k_6$. However, this cannot be done by a plot of $1/\tau_2$ vs. $4[A_{580}]$ (as in Figure 8) inasmuch as both the dimer and monomer semiquinone absorb at 580 nm. Indeed, because of the apparent slowness of radical disproportionating as measured by the temperature-jump (preceding section), most of the radical must be dimerized at these concentrations. Therefore, the greater part of the absorbance at 580 nm is due to the dimer as represented below.

$$A_{580} [\text{FH}^\cdot]_2 = 2\varepsilon_{\text{FH}^\cdot} [\text{FH}^\cdot]_2$$

We can make use of the equilibrium relationship between dimer and monomer and solve for the concentration of monomer in terms of $A_{580}$ as shown below:

$$[\text{FH}^\cdot] = \frac{K_{56}^{-1}}{2\varepsilon_{\text{FH}^\cdot}} \sqrt{A_{580}}$$

Therefore a plot of $1/\tau_2$ vs. $\sqrt{A_{580}}$ should be linear with a slope proportional to $k_6$ and intercept equal to $k_5$ (Figure 19). The exact concentration of $[\text{FH}^\cdot]$ cannot be determined and therefore $k_6$ cannot be calculated. However, the intercept does yield $k_5 (1.5 \times 10^3 \text{sec}^{-1})$ and if this is combined with $k_6 (3 \times 10^3 \text{sec}^{-1})$ as determined by flash
Figure 19. A plot of $\frac{1}{T}$ vs. $\sqrt{A_{580}}$ for the determination of the kinetics of FMN semiquinone dimerization (pH 4.5).
photolysis) the equilibrium constant between monomer and dimer may be estimated \( k_{56} = k_5/k_6 = 5 \times 10^{-7} \text{ M.} \)

**Kinetics and Thermodynamics of the Binding of Flavin Analogs to the Shethna Apoprotein**

In order to better understand the nature of the flavin-protein interaction, the kinetics and thermodynamics of binding of several flavin analogs to the Shethna apoprotein were determined. The Shethna flavoprotein is particularly useful in this type of experiment because of its high stability, low molecular weight, and possession of only one flavin binding site. The FMN cofactor is easily resolved and can be subsequently recombined with complete renaturation as evidenced by C. D. and redox properties (Edmondson and Tollin 1971, p. 113). The apoprotein will very efficiently bind other flavin analogs as well. Previous work in our laboratory has demonstrated the importance of the ribityl-phosphate side chain and N-5 ring nitrogen of FMN in determining redox properties (Vaish and Tollin 1971; Edmondson and Tollin 1971, p. 124). It was of interest, therefore, to determine the role of the phosphorylated side chain and N-5 nitrogen in the binding process.

The six flavin derivatives that were used are represented in Figure 1. Riboflavin and lumiflavin differ from FMN by the absence of phosphate and ribityl-phosphate group, respectively. They therefore provide a probe for the elucidation of the side chain role. Riboflavin-5'-sulfate differs from FMN by the replacement of phosphate by sulfate. It thus can give an indication of the specificity of the protein for phosphate. In the deaza derivatives, the N-5 ring nitrogen
has been replaced by carbon, and thus they provide an opportunity to study the role of this position in binding.

**Association Constants and Equilibrium Thermodynamics**

By titration of flavin fluorescence with apoprotein the association constants ($K_a$) and and Gibbs free energies ($\Delta G^\circ$) of binding for all analogs were determined. A description of the method is given by Edmondson and Tollin (1971, p. 124). In addition, the titrations of FMN, riboflavin, and lumiflavin were carried out at two temperatures so that the enthalpy ($\Delta H^\circ$) and entropy ($\Delta S^\circ$) of binding could be calculated (Table 6).

Inspection of Table 6 shows:

(a) the phosphorylated side chain significantly enhances binding, suggesting a strong charge-charge interaction between flavin and protein (comparing FMN and deazaFMN with riboflavin, lumiflavin, and deazariboflavin). A similar enhancement is found in comparing FMN with other nonphosphorylated analogs (Edmondson and Tollin 1971, p. 124).

(b) riboflavin sulfate binds only slightly less strongly than FMN, showing that the negatively charged sulfate substitutes well for phosphate.

(c) the substitution of carbon for nitrogen at the N-5 position decreases the binding energy by about 1 Kcal/mole, indicating the possible loss of a hydrogen bond at that position (comparing FMN with deazaFMN and riboflavin with deazariboflavin).
Table 6. Equilibrium binding constants of flavin analogs to the Shethna apoprotein

<table>
<thead>
<tr>
<th>Flavin</th>
<th>( k_a (24^\circ C) )</th>
<th>( K_a (11^\circ C) )</th>
<th>( \Delta H^o ) (kcal/mole)</th>
<th>( \Delta G_{24^\circ}^o ) (kcal/mole)</th>
<th>( \Delta S^o ) (cal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>( 1.7 \times 10^8 )\textsuperscript{a}</td>
<td>( 2.4 \times 10^8 )</td>
<td>-4.0</td>
<td>-11.1</td>
<td>23.9</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>( 1.8 \times 10^6 )\textsuperscript{a}</td>
<td>( 3.0 \times 10^6 )</td>
<td>-6.6</td>
<td>-8.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Luminflavin</td>
<td>( 2.2 \times 10^5 )\textsuperscript{b}</td>
<td>( 3.7 \times 10^5 )</td>
<td>-6.2</td>
<td>-7.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Riboflavin-SO\textsubscript{4}</td>
<td>( 6.3 \times 10^7 )</td>
<td></td>
<td></td>
<td>-10.6</td>
<td></td>
</tr>
<tr>
<td>DeazaFMN</td>
<td>( 4.0 \times 10^7 )</td>
<td></td>
<td></td>
<td>-10.3</td>
<td></td>
</tr>
<tr>
<td>Deazariboflavin</td>
<td>( 4.2 \times 10^5 )</td>
<td></td>
<td></td>
<td>-8.0</td>
<td></td>
</tr>
</tbody>
</table>

\*a.* Agree well with \( K's \) calculated by Edmondson and Tollin (1971, p. 124).

(d) the ribityl side chain contributes about 1 Kcal mole to the binding energy, suggesting a possible hydrogen bond between the side chain and protein (comparing riboflavin with lumiflavin). Similar results have been obtained by Edmondson and Tollin (1971, p. 124).

Inspection of the thermodynamic constants reveals a larger free energy and smaller enthalpy of binding for FMN than for either riboflavin or lumiflavin. This is reflected in a much larger entropy change for FMN. This large entropy term could possibly result from a protein rearrangement following binding of FMN, or from desolvation of the FMN phosphate group upon binding, or, perhaps, from both processes (see below). Similar examples of this behavior have been found in model enzyme systems using cyclodextrins to approximate the active site of a protein for the binding of polar and solvated ligands (Van Etten et al. 1967).

**Kinetics of Analog Binding**

For the binding of flavin analogs to apoprotein, the kinetics should be simple second order, barring any subsequent first order conformational steps. If such is the case, in a temperature-jump experiment only one relaxation should be seen. The relaxation time for the mechanism below [equation (41)],

\[
F + P \xrightleftharpoons[k_1][k_2] FP
\]  

should obey the following equation (42):

\[
\frac{1}{\tau} = k_2 [F + P] + k_1
\]  

(42)
where $k_2$ and $k_1$ are the forward and back rate constants, while $\bar{F}$ and $\bar{P}$ are the new equilibrium concentrations of free flavin and apoprotein. Therefore, if the above mechanism is operative, a plot of the reciprocal relaxation time vs. $[\bar{F} + \bar{P}]$ will be linear. From such a plot, the slope and intercept yield the forward ($k_2$) and back ($k_1$) rate constants, respectively.

However, if a temperature-jump experiment shows multiple relaxation times (that are not well separated) the kinetic analysis becomes more complicated. If two relaxations are observed for the binding of a flavin analog to apoprotein, it is reasonable to assume that these reflect a bimolecular step where flavin ($F$) and apoprotein ($P$) come together to form an initial flavoprotein species ($X$), followed by a rearrangement to form a different species ($FP$).

The mechanism for such a process can be described by equation (43). \[
\begin{align*}
\text{\textit{F + P $\xleftarrow{k_2} \xrightarrow{k_1} \xleftarrow{k_4} \xrightarrow{k_3} \text{FP}}$
\end{align*}
\]

Linearization of the independent rate equations for the above mechanism leads to two expressions involving the relaxation times [equations (44) and (45)].

\[
\frac{1}{\tau_1} + \frac{1}{\tau_2} = k_2[\bar{F} + \bar{P}] + k_1 + k_3 + k_4 \tag{44}
\]

\[
\frac{1}{\tau_1} \times \frac{1}{\tau_2} = \frac{k_3k_{12}[\bar{F} + \bar{P}]}{1 + K_{12}[\bar{F} + \bar{P}]} + k_4 \tag{45}
\]
The two relaxation times can be determined as a function of free flavin and apoprotein concentrations by analog computer fitting of the experimental data, as described earlier. It is possible from a knowledge of $\frac{1}{\tau_1}$ and $\frac{1}{\tau_2}$, as a function of concentration $[\bar{F} + \bar{P}]$, and using the previously determined association constant for the overall binding process, to determine all four rate constants.

Experimentally, for the nonphosphorylated derivatives (riboflavin, lumiflavin, deazariboflavin) we see only a single relaxation. This is verified by the linearity of a plot of log $\Delta A$ (signal) vs. time. The relaxation times are determined from the slope of such a plot. A typical relaxation trace (riboflavin binding) along with its log plot is shown in Figure 20. As indicated above, the rate constants can be calculated from plots of $\frac{1}{\tau}$ vs. the free concentrations of flavin analog and apoprotein (determined from the association constants). Plots for lumiflavin and riboflavin are shown in Figures 21 and 22 along with the calculated rate constants. The kinetics for deazariboflavin were obtained by determining $\frac{1}{\tau}$ at only a few concentrations and then calculating the rate constants using the already determined association constants and equations (46) and (47).

$$\frac{1}{\tau} = k_2[\bar{F} + \bar{P}] + \frac{k_2}{K_a}$$  \hspace{1cm} (46)

$$K_a = \frac{k_2}{k_1}$$  \hspace{1cm} (47)

Using simple mixing experiments, Edmondson and Tollin (1971, p. 124) have shown that the kinetics of binding of FMN to the Shethna apoprotein
Figure 20. Plot of log $\Delta A$ vs. time for the determination of the relaxation time for the binding of riboflavin ($2 \times 10^{-5}$ M) to the Shethna apoprotein ($2 \times 10^{-5}$ M). $0.1$ M phosphate buffer (pH 7.5); $0.1$ M KNO$_3$. 
Figure 21. Plot of $\frac{1}{t}$ vs. $[\overline{F} + \overline{P}]$ for riboflavin binding to the Shethna apoprotein.

$$k_2 = 8.5 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$$

$$k_1 = 0.5 \text{ sec}^{-1}$$
Figure 22. Plot of $\frac{1}{T}$ vs. $[\bar{F} + \bar{P}]$ for lumiflavin binding to the Shethna apoprotein.

$$k_2 = 4.0 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$$
$$k_1 = 1.8 \times 10^2 \text{ sec}^{-1}$$
are simple second order. Stopped-flow experiments verify this behavior. However, this does not preclude a multiple step process, if the rate limiting step was second order and a faster first order step followed. Temperature-jump experiments show that the kinetics are indeed not simple second order. Two separate relaxations are observed, suggesting a mechanism of the type indicated in equation (43).

In Figure 23 is shown a typical relaxation trace obtained for the binding of FMN. Also, in Figure 23 a cooling time blank is shown in order to demonstrate that the second slower transient is not simply a return to the original equilibrium temperature but is actually due to a binding process. Similar results were obtained using phenol red and the riboflavin plus apoprotein systems to determine cooling times. A comparison of this curve with that shown in Figure 20 clearly indicates a mechanistic difference. It was found (see later in this section) that the initial downward deflection (fluorescence decrease) was concentration-independent whereas the following upward deflection (fluorescence increase) was concentration-dependent. From this behavior it is proposed that upon mixing FMN and apoprotein, there is first formed a flavoprotein species X, which subsequently can rearrange to form a second species (FP). To account for the observed relaxation spectrum, we propose the following situation. At equilibrium, all four species are present (F, P, X, and FP). Upon rapidly raising the temperature, the equilibrium concentration of X is forced to decrease in both directions, forming more F and P and also more FP. It is reasonable to assume that any initial complex of flavin and apoprotein (X), in which
Figure 23. Relaxation trace for the binding of FMN to the Shethna apoprotein together with FMN cooling time blank.

The rapid downward deflection (fluorescence decrease) in the lower trace is due to the fact that the FMN fluorescence yield is lower at the higher temperature; this quenching occurs within the heating time of the instrument.
the flavin is not well buried within the protein, would show more flavin fluorescence than one in which the flavin is more completely buried (FP). This assumption would satisfactorily account for the observed fluorescence behavior following a temperature perturbation. From the directions of the fluorescence changes, it can be concluded that the bimolecular binding step is exothermic and the monomolecular step is endothermic. The latter would be consistent with a protein conformational change requiring significant disruption of secondary and tertiary structure. Edmondson and Tollin (1971, p. 133) have suggested that there is a change in protein structure upon the binding of flavin, based on a comparison of the far U.V. CD spectra of the apo and holo forms of the Shethna flavoprotein.

Similar relaxation behavior (to that found with FMN) is observed for 5-deazaFMN (Figure 24), indicating that the N-5 nitrogen does not play a key role in this two step mechanism. It should be noted, however, that relaxation is somewhat faster for deazaFMN than for FMN which may suggest a small degree of cooperation between the phosphate group and this position in the binding process. A similar conclusion is arrived at on the basis of stopped-flow experiments (see below). Temperature-jump experiments with riboflavin sulfate were not successful due to partial hydrolysis of the sulfate group to form riboflavin during the course of the experiment.

It is interesting that temperature-jump experiments on *P. elsdenii* flavodoxin showed the same relaxation behavior as with the Shethna flavoprotein (Figure 25). Although a concentration dependence of the relaxation times were not performed, the general shape of the
Figure 24. Relaxation traces for the binding of 5-deazaFMN to the Shethna apoprotein at pH 7.5.

The same sample ($6 \times 10^{-5}$ M both in analog and protein) was used for both traces. The sample also contains 0.05 M phosphate buffer and 0.1 M KNO$_3$. 
Figure 25. Relaxation traces for binding of FMN to *P. elsdenii* flavodoxin apoprotein at pH 6.8.

The same sample (8 x 10^{-5} M both in flavin and apoprotein) was used for both traces. The sample also contains 0.025 M phosphate buffer and 0.1 M KNO₃.
relaxation curves are the same and thus the mechanism of FMN binding is probably similar.

In Table 7 we have tabulated the forward and reverse rate constants for the binding of the flavin analogs to the apoprotein. It should be noted that the rate constants for FMN are apparent rate constants, where

\[ k_2 \text{ app} = \frac{k_2 k_3}{k_1 + k_3} \] (48)

and

\[ k_1 \text{ app} = \frac{k_1 k_4}{k_1 + k_3} \] (49)

These are derived from the true rate constants by using a "steady state" approximation for the concentration of \( X \). In addition, the rate constants for FMN, 5-deazaFMN, and riboflavin sulfate were determined by the stopped-flow method, while the temperature-jump method was used for riboflavin, lumiflavin, and 5-deazariboflavin.

Inspection of Table 7 shows that the nonphosphorylated derivatives come off the protein much more rapidly than do the phosphorylated derivatives. If there is a phosphate-induced protein conformational change subsequent to the initial flavin-protein contact which leads to more complete burying of the FMN molecule within the protein, as indicated by the temperature-jump behavior, this could explain the slower rate of leaving of the phosphorylated derivatives. In addition, the slower binding rate for the phosphorylated analogs suggests that the phosphate may be buried within the binding site. Recent
Table 7. Rate constants at 24°C $[F + P \xrightarrow{k_2} \text{FP}]$.

<table>
<thead>
<tr>
<th>Analog</th>
<th>$k_2$ (M$^{-1}$sec$^{-1}$)</th>
<th>$k_1$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>$2.0 \times 10^5$</td>
<td>$1.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>$8.5 \times 10^5$</td>
<td>0.5</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>$4.0 \times 10^7$</td>
<td>$1.8 \times 10^2$</td>
</tr>
<tr>
<td>RbF-SO$_4$</td>
<td>$5.6 \times 10^5$</td>
<td>$8.8 \times 10^{-2}$</td>
</tr>
<tr>
<td>DeazaFMN</td>
<td>$4.1 \times 10^4$</td>
<td>$1.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>DeazaRbF</td>
<td>$2.4 \times 10^5$</td>
<td>0.6</td>
</tr>
</tbody>
</table>
crystallographic work by Dr. Martha Ludwig (1971) has shown that in a
Clostridial flavodoxin the flavin (FMN) is buried in a cleft in the
protein. A similar situation also exists for the Desulfovibrio vulgaris
flavodoxin (Jensen 1972). Edmondson and Tollin (1971, p. 133) have
reached similar conclusions about the binding site in the Shethna flavo-
protein on the basis of chemical studies. It is thus possible that the
phosphate group triggers the closing of this cleft subsequent to the
initial binding process. The ribityl chain must also be at least
partly buried within the protein inasmuch as lumiflavin leaves the bind-
ing site much more rapidly than does riboflavin. This conclusion is
also consistent with the crystallographic studies on the FMN proteins.

The kinetics of binding of the deaza analogs parallel their FMN
and riboflavin counterparts, so it appears that the N-5 ring nitrogen
contributes little to the overall mechanism of binding. However, the
decrease in binding rate for 5-deazaFMN compared with FMN suggests some
cooperativity between the phosphate and N-5 nitrogen during the initial
binding process (the temperature-jump results indicate that a similar
situation exists during the subsequent first order step, i.e., the pro-
tein conformational rearrangement proceeds faster with the deaza ana-
log). It is possible that for the binding of FMN the coenzyme is in a
folded conformation in which the N-5 nitrogen and the phosphate group
present themselves simultaneously to the apoprotein, thus effecting a
rate enhancement. This would imply that the flavin binding site within
the apoprotein has a complementary shape. In addition, the faster rate
at which riboflavin sulfate leaves the protein indicates that the
sulfate may not be as effective as the phosphate in causing the protein to rearrange.

From the concentration dependence of the temperature-jump relaxation curves for FMN binding, it is possible to evaluate all four rate constants and the equilibrium constants for both steps. Three experimental traces and the analog computer curves which best fit the data, along with the plots of log ΔS vs. t for the two computer-derived exponentials, are shown in Figures 26-28. Because of the poor signal-to-noise ratio in these experiments, it was not possible to vary concentration over a wide range. In Figure 29 a plot of \( \frac{1}{\tau_1} + \frac{1}{\tau_2} \) vs. \([F + P]\) is given from which the rate constants \(k_2\) and the sum \((k_1 + k_3 + k_4)\) are determined. As \(\frac{1}{\tau_1}\) is constant, and \(\frac{1}{\tau_2}\) at zero \([F + P]\) concentration is determined by the above plot, \(k_4\) can be calculated from

\[
\frac{1}{\tau_1} \times \frac{1}{\tau_2} = k_4 \text{ at } [F + P] = 0
\]

Since the overall association constant is known and equal to \(k_2k_3/k_1k_4\), the ratio of \(k_3\) to \(k_1\) can then be calculated. Combining this with knowledge of the sum \((k_1 + k_3 + k_4)\) as determined from the above plot, \(k_3\) and \(k_1\) can be calculated. Since all four rate constants are now known, the individual equilibrium constants \(k_{12} = k_2/k_1\) and \(K_{34} = k_3/k_4\)

1. This analysis was done by holding constant the exponential time constant for the initial fluorescence decrease and varying the time constant for the slower fluorescence increase. Only in this manner could the data be fit. Transients were essentially equal in amplitude such that the net fluorescence change was zero.
Figure 26. Plot of log $\Delta A$ vs. time for the determination of $\frac{1}{\tau_1}$ and $\frac{1}{\tau_2}$ for the Shethna flavoprotein ($10^{-4}$ M) at pH 7.5.

Experimental and analog computer resolution traces superimposed.

$X = \frac{1}{\tau_1} = 0.137$ sec$^{-1}$

$O = \frac{1}{\tau_2} = 0.100$ sec$^{-1}$
Figure 27. Plot of log $\Delta A$ vs. time for the determination of $\frac{1}{\tau_1}$ and $\frac{1}{\tau_2}$ for the Shethna flavoprotein (7.6 x $10^{-5} M$) at pH 7.5.

Experimental and analog computer resolution traces superimposed.

\[ X = \frac{1}{\tau_1} = 0.137 \text{ sec}^{-1} \]
\[ 0 = \frac{1}{\tau_2} = 0.090 \text{ sec}^{-1} \]
Figure 28. Plot of log $\Delta A$ vs. time for the determination of $\frac{1}{\tau_1}$ and $\frac{1}{\tau_2}$ for the Shethna flavoprotein ($5.2 \times 10^{-5}$ M) at pH 7.5.

Experimental and analog computer resolution traces superimposed.

$X = \frac{1}{\tau_1} = 0.137 \, \text{sec}^{-1}$

$O = \frac{1}{\tau_2} = 0.080 \, \text{sec}^{-1}$
Figure 29. Plot of $\frac{1}{\tau_1} + \frac{1}{\tau_2}$ vs. $[\bar{F} + \bar{P}]$ for the determination of the rate constants for the binding of FMN to the Shethna apoprotein (pH 7.5, 10°C).
can be calculated. The results are shown in Table 8. In spite of the small range over which concentration was varied, all kinetic constants are believed to be at least within an order of magnitude or better of the true rate constants.

The initial association constant ($K_{12}$) is seen to be quite close to the association constant for riboflavin (same order of magnitude), indicating that the phosphate group does not contribute greatly to the energetics of this step (although it does contribute to both the on and off rates). Thus, the equilibrium constant for the second step ($K_{34}$) is sufficient to account for the major portion of the increase in the association constant of FMN over that of riboflavin. These results are consistent with a phosphate-triggered protein rearrangement following an initial phosphate-protein interaction.

It should be noted that there is a slight sigmoidicity in the initial portion of all of the FMN relaxation curves. This could be due to yet another step in the binding process that is fast and corresponds to a fluorescence increase. The duration and magnitude of this relaxation did not allow for analog computer resolution and so it was neglected in the calculations. It is also possible, although unlikely, that this sigmoidicity is artifactual and due perhaps to the presence of a small contamination of riboflavin. (If a small amount of bound riboflavin was present, this would result in a fast fluorescence increase upon heating, as observed.)
Table 8. Rate and equilibrium constants for the binding of FMN to the Shethna apoprotein.

<table>
<thead>
<tr>
<th>Rate constants (9°C)</th>
<th>Equilibrium constants (9°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_2 = 5.3 \times 10^4 \text{ M}^{-1} \text{sec}^{-1}$</td>
<td>$K_{12} = 6.5 \times 10^6 \text{ M}^{-1}$</td>
</tr>
<tr>
<td>$k_1 = 8.1 \times 10^{-3} \text{ sec}^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_3 = 1.6 \times 10^{-1} \text{ sec}^{-1}$</td>
<td>$K_{34} = 37$</td>
</tr>
<tr>
<td>$k_4 = 4.2 \times 10^{-3} \text{ sec}^{-1}$</td>
<td></td>
</tr>
</tbody>
</table>

pH 7.0 in 0.025 M phosphate buffer
Determination of Activation Parameters

For the binding of FMN, riboflavin, and lumiflavin, kinetics were determined at two temperatures in order to obtain an estimate of the Arrhenius energy and entropies of activation. The results are tabulated in Table 9. For FMN the forward and back rate constants are the apparent constants \[ k_{2\text{app}} = \frac{k_2 k_3}{(k_1 + k_3)} \text{ and } k_{1\text{app}} = \frac{k_1 k_4}{(k_1 + k_3)} \]. The use of these constants for a comparison with the activation parameters for riboflavin and lumiflavin would seem more valid since we wish to describe the changes in going from initial to final states.

Of particular interest is the comparison of activation entropies in both the forward and reverse directions. The entropy changes for FMN are quite different from those for riboflavin and lumiflavin. Although the interpretation of activation parameters for large molecules is difficult, it is probably safe to say that the large differences are due mainly to the protein-phosphate and solvent-phosphate interactions. In the activated complex for initial binding step, partial removal of solvent from the charged phosphate group, and perhaps from the protein binding site as well, could account for an increase in entropy. In the reverse process, resolvation and a loosening of protein structure during the conformational change could act in opposite directions to yield a small net activation entropy. According to this interpretation, the larger negative entropies of the dissociation step for riboflavin and lumiflavin would reflect mainly solvation effects inasmuch as a protein conformational rearrangement does not occur. The much smaller entropy changes in the association steps for riboflavin
Table 9. Arrhenius energy and entropy of activation determined by kinetics at two temperatures.

<table>
<thead>
<tr>
<th></th>
<th>Forward rate constants</th>
<th></th>
<th>Reverse rate constants</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F + P \rightarrow FP$</td>
<td></td>
<td>$FP \rightarrow F + P$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$k_2(297^\circ)$</td>
<td>$k_2(283^\circ)$</td>
<td>$E_A$</td>
<td>$\Delta S_2^0$</td>
</tr>
<tr>
<td></td>
<td>M$^{-1}$sec$^{-1}$</td>
<td>M$^{-1}$sec$^{-1}$</td>
<td>kcal/mole</td>
<td>cal/mole</td>
</tr>
<tr>
<td>FMN</td>
<td>$2.0 \times 10^5$</td>
<td>$5.3 \times 10^4$</td>
<td>12.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>$8.9 \times 10^5$</td>
<td>$3.9 \times 10^5$</td>
<td>9.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>$4.0 \times 10^7$</td>
<td>$2.2 \times 10^7$</td>
<td>7.1</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$k_1(297^\circ)$</td>
<td>$k_1(283^\circ)$</td>
<td>$E_A$</td>
<td>$\Delta S_1^0$</td>
</tr>
<tr>
<td></td>
<td>sec$^{-1}$</td>
<td>sec$^{-1}$</td>
<td>kcal/M</td>
<td>cal/M</td>
</tr>
<tr>
<td>FMN</td>
<td>$1.2 \times 10^{-3}$</td>
<td>$2.1 \times 10^{-4}$</td>
<td>20.9</td>
<td>-0.8</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.49</td>
<td>0.13</td>
<td>15.9</td>
<td>-6.4</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>180</td>
<td>59</td>
<td>13.3</td>
<td>-3.4</td>
</tr>
</tbody>
</table>
and lumiflavin (as compared with FMN) could be interpreted as indicating that for these analogs the solvation in the activated complex is predominantly that of the free ligand and protein. The necessity for appreciable desolvation during FMN binding could partially account for the relative magnitudes of the on-constants for the various species, i.e., lumiflavin and riboflavin > FMN. Such a mechanism is also consistent with the somewhat larger rate constant observed for riboflavin sulfate (Table 7), inasmuch as the sulfate group has one less negative charge than does the phosphate and hence solvent removal should be more easily accomplished. The rather large difference observed between the on-constants of riboflavin and lumiflavin is probably due mainly to steric effects.

Additional Experiments with 5-Deaza Flavin Analogs

Redox Properties of 5-Deaza Flavin Analogs

It has been shown previously that the nitrogen in the 5-position of the isoalloxazine ring is involved in determining some of the redox properties of free flavins (Holmstrom 1964, Vaish and Tollin 1971). In addition, the 5-position has been shown to have a high percentage of the unpaired spin density in the flavin semiquinone as calculated from ESR measurements (Guzzo and Tollin 1964). For these reasons, this position is considered to be a possible site for the transfer of electrons to and from free and protein bound flavin coenzymes. It is thus of interest to determine the redox properties of 5-deazaFMN and to compare them with FMN.
Edmondson, in collaborative experiments (Edmondson, Barman, and Tollin 1972) has shown that the dithionite reduction of deazaFMN is qualitatively similar to ordinary FMN, but proceeds much more slowly. In addition, he has determined that the rate of $O_2$ oxidation of the fully reduced deaza analog is also slower ($t_{1/2} = 180$ min). This can be compared with a half-life of less than one second for fully reduced FMN (Gibson and Hastings 1962). Furthermore, he has determined that the deazaflavin analogs are photoreduced in the presence of EDTA at a rate which is at least as fast as that of ordinary flavins. Thus, in general, the 5-deaza analogs have redox properties that qualitatively parallel those of ordinary flavins.

The ability of the deaza analogs to be photoreduced implies the intermediacy of triplet states and semiquinone radicals (Vaish and Tollin 1970). In order to confirm this, a flash photolysis study was performed. In Figure 30, flash-induced difference spectra are shown for 5-deazariboflavin in the presence and absence of 3,4-dimethylphenol (Vaish and Tollin 1970). The transient which has an absorption maximum at approximately 500 nm is undoubtedly the neutral semiquinone radical [the maximum wavelength for the corresponding FMN semiquinone is about 560 nm; thus, the spectral shift is similar in magnitude to what was observed for the oxidized form (Edmondson et al. 1972)]. This identification was confirmed by a flash experiment using EDTA as the reducing agent (Vaish and Tollin 1971). Second order decay kinetics were observed (Figure 31) and the sample was bleached after about ten flashes. This is consistent with radical disproportionation. A second order
Figure 30. Flash-induced difference spectra for 5-deazariboflavin in 0.025 M acetate buffer, pH 4.8.

- X — X [flavin] = 8 x 10^6 M; [3,4-dimethylphenol] = 1 x 10^-3 M
- O — O [flavin] = 1.5 x 10^-5 M
Figure 31. Second order plot of decay of flash-induced transient at 500 nm for 5-deazariboflavin in 0.025 M acetate buffer, pH 4.8, containing 0.50 M EDTA.
rate constant of $9.1 \times 10^9 \, M^{-1} \, \text{sec}^{-1}$ was calculated, which is about eight times that observed with riboflavin (Land and Swallow 1969), about three times that for FMN, and slightly larger than for lumiflavin (Vaish and Tollin 1971). This calculation assumes that the absorptivity of the deaza radical is the same as that for the flavin radical (Land and Swallow 1969). Qualitatively, the radical yield was somewhat smaller for the deaza analog than for lumiflavin. As with ordinary flavins, sample bleaching by the flash was considerably retarded when phenol was used as the reductant (Vaish and Tollin 1970). These experiments confirm that a neutral radical form can be generated with the deazaflavins and that disproportionation of the radical can occur to form a fully reduced compound.

The transient which has an absorption maximum at approximately 675 nm is most probably the triplet state (Vaish and Tollin 1970, Knowles and Roe 1968). This is confirmed by the fact that the signal was not observed in the presence of reducing agents (Figure 30), presumably because of photochemical quenching. The decay of the triplet is of mixed order. The life-time and yield of the deazaflavin triplet are comparable to those for ordinary flavins. This is consistent with the observed rapid rate of photoreduction.

Electron spin resonance experiments were performed upon mixtures of equal amounts of oxidized and fully reduced 5-deaza FMN ($10^{-3} \, M$ in each). No measurable signal was found that would indicate the presence of a free radical. In addition, there was no long wavelength (700-900 nm) absorption band detected in these mixtures due to the presence
of a complex of oxidized and fully reduced deazaFMN. These species are
easily detectable under corresponding conditions with FMN (Gibson et
al. 1962). Thus, radical disproportionation is essentially irreversi­
ble with the deaza analogs. It is somewhat surprising that no complex
is formed; this points to an important role for the 5-position nitrogen
in the stabilization of this species, perhaps via a key hydrogen bond.

Circular Dichroism Studies on Free
and Protein-bound 5-DeazaFMN

Circular dichroism spectroscopy provides a measure of ring-side
chain interaction in the free coenzyme, and this interaction plus ring-
protein interactions in the protein-bound form (Tollin 1968, Edmondson
and Tollin 1971, p. 113). It was thus of interest to obtain these
spectra for the 5-deaza species. In Figure 32 is shown the CD spectra
of free 5-deazaFMN and of the 5-deazaFMN-Shethna apoprotein complex.
The ellipticities of the free analog are approximately the same as
those found for free FMN (Tollin 1968, Edmondson and Tollin 1971, p.
113). A major difference occurs in the sign of the long wavelength
CD band, which is positive for the 5-deaza derivative and negative for
FMN (Tollin 1968). This indicates that, on the average, the relative
positions of the ring and side chain are different in the two species.
It is noteworthy that the major CD band in both flavins appears at ap­
proximately the same wavelength (ca. 340 nm). Thus, this band, which
is not observable in the ordinary absorption spectrum (Tollin 1968),
must not have an appreciable component of the transition moment at the
5-position.
Figure 32. CD spectra of free 5-deazaFMN (bottom curve) and of the 5-deazaFMN-Shethna apoprotein complex (top curve) in 0.025 M phosphate buffer, pH 7.0.
The CD spectrum of the 5-deazaFMN protein is quite similar in general shape and band signs to that of the native Shethna flavoprotein (Edmondson and Tollin 1971, p. 113). This demonstrates that the 5-deazaFMN is bound in a specific manner to the apoprotein and that the overall conformational characteristics of the flavin analog in its binding site are not too different from those of the normal coenzyme. However, the magnitudes of the ellipticities are considerably lower for the deaza analog (ca. 40% for the longest wavelength band and ca. 33% for the most intense shorter wavelength band). Inasmuch as the long wavelength band monitors mostly the ring-side chain interaction (Tollin 1968, Edmondson and Tollin 1971, p. 113) and the shorter bands the ring-protein interaction (Edmondson and Tollin 1971, p. 113; Tollin and Edmondson 1971), both of these characteristics can be said to be affected by the replacement of nitrogen by carbon.

Redox Properties of the 5-DeazaFMN Apoprotein Complex

The 5-deazaFMN protein is rapidly reduced by excess dithionite (1 mg/ml) at pH 7.0, but unlike the native Shethna protein (Edmondson and Tollin 1971, p. 133), the reaction proceeds directly to the fully reduced form. In addition, there was no detectable half-reduced intermediate (Figure 33). Furthermore, not all of the protein is reduced under these conditions, which implies a thermodynamic barrier to full reduction.

The rate of \( \text{O}_2 \) oxidation of the dithionite-reduced deaza protein was also determined. The half-time of oxidation was calculated
Figure 33. Dithionite reduction and air oxidation of 5-deazaFMN-Shethna apoprotein complex in 0.025 M phosphate buffer, pH 7.0.

- oxidized form
- dithionite reduced
- 25 min. after O$_2$ added
- 50 min. after O$_2$ added
- 80 min. after O$_2$ added
- 140 min. after O$_2$ added
to be 70 minutes. Surprisingly, this is faster than for the free analog. Again, upon spectrally monitoring the reoxidation, no measurable semiquinone was observed. This is not true of the native protein where reoxidation gives essentially only semiquinone protein with subsequently a very slow (days) return to the fully oxidized state (Edmondson and Tollin 1971, p. 133).

The deazaFMN protein was also reduced photochemically in the presence of EDTA. Again, unlike the native protein, only the fully reduced form was produced with no detectable semiquinone intermediate. Because the photochemical reduction is slow and most probably is a one-electron process, this suggests that the deazaflavoprotein semiquinone disproportionates. As in the dithionite reduction, the photochemically reduced deazaflavoprotein completely reoxidized with air with no measurable semiquinone formation. In addition the photochemical reduction would only proceed to about 50% completion, just as was observed in the dithionite reduction.

The above results demonstrate quite clearly that the N-5 position is crucial in order for the protein to stabilize the semiquinone form of the flavoprotein. This is particularly emphasized by the fact that whereas 3-methylFMN and isoFMN bind to the Shethna apoprotein with approximately the same strength as does 5-deazaFMN (Edmondson and Tollin 1971, p. 124) both of these analogs generate semiquinones upon either dithionite reduction or photoreduction (Edmondson and Tollin 1971, p. 133). Although the deazaflavin radical is somewhat less stable than the normal flavin species (as judged by the faster disproportionation rate),
this does not seem to be sufficient to account for the complete lack of semiquinone formation in the analog protein.

**Thermodynamics and Kinetics of the Reduction of the Shethna Flavoprotein**

**Determination of Reduction Potentials for the Shethna Flavoprotein**

The two one-electron reduction potentials were determined by the methods previously described. The sequence of reduction at pH 8.2 is described by equations (51) and (52).

\[ \begin{align*}
E_1^o & \quad \text{FP} + [H^+] \rightleftharpoons \text{FPH^+} \\
E_2^o & \quad \text{FPH^+} + [H^+] \rightleftharpoons \text{FPH}^- + H^+ 
\end{align*} \]

In Figure 34 the course of reduction as monitored by absorption spectra is shown upon going from the oxidized to the semiquinone flavoprotein. The absorption at 450 nm is decreased while a broad absorption (due to the semiquinone) with peaks at 580 nm and 615 nm appears as the potential becomes increasingly negative. There is a well-defined isosbestic point at 500 nm, showing that only two species are present in equilibrium. In Figure 35 is shown a plot of log Ox/Red vs. E for the determination of \( E_1^o \). The intercept at log Ox/Red = 0 yields a value of \( E_1^o \) of +50 mv. The slope corresponds to a one-electron process.
Figure 34. Coulometric reduction of the Shethna flavoprotein to the semiquinone form.

pH 8.2; 0.1 M tris.
Figure 35. Plot of log ox/red vs. E for the determination of the Shethna flavoprotein oxidized/semiquinone reduction potential at pH 8.2.
In Figure 36 the course of reduction in going from the semiquinone to the hydroquinone flavoprotein is shown. Because of the interference in absorption at these negative potentials by methyl viologen (5 mole percent), no clear isosbestic point is seen. As the cell potential became increasingly negative, the 580 nm absorption disappeared. The plot of log Ox/Red vs. E is linear and corresponds to a one-electron reduction (Figure 37). The intercept at log Ox/Red = 0 yields a reduction potential ($E^0_2$) of -495 mv.

Since the Shethna flavoprotein has properties which are similar to the flavodoxins from bacteria, it is of interest to compare the reduction potentials for this protein with those of the various flavodoxins. Earlier work (Hinkson and Bulen 1967, Benemann et al. 1969, Cusanovich and Edmondson 1971) had shown that the Shethna flavoprotein would not substitute for ferredoxins in the photosynthetic reduction of NADP+ by spinach chloroplasts. However, more recent work by Van Lin and Bothe (1972) has shown that the Shethna flavoprotein can indeed substitute for ferredoxin in NADP+ - reduction and thus this protein should be classified as a flavodoxin. In light of this, the comparison of reduction potentials becomes even more significant (Table 10).

Inspection of Table 10 shows that the Shethna flavoprotein possesses by far the most positive first reduction potential of all of the flavodoxins. Indeed, this potential ranges from $\approx 150$ to $\approx 290$ mv more positive than any of the other flavodoxins and accounts well for the unusually high stability of the Shethna semiquinone to oxidation. On the other hand, the second reduction potential is the most negative
Figure 36. Potentiometric reduction of the Shethna flavoprotein semiquinone to the hydroquinone form.

pH 8.2; 0.1 M tris buffer.
Figure 37. Plot of log ox/red vs. E for the determination of the Shethna flavoprotein semiquinone/hydroquinone reduction potential at pH 8.2.
Table 10. Reduction potentials of flavodoxins.

<table>
<thead>
<tr>
<th>Organism - Azotobacter</th>
<th>Clostridium pasteurianum</th>
<th>Clostridium MP</th>
<th>P. elsdenii</th>
<th>E. coli</th>
<th>D. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_1^0$ oxid./semiq.</td>
<td>+50 mv</td>
<td>-132 mv</td>
<td>-92</td>
<td>-115</td>
<td>-240</td>
</tr>
<tr>
<td>$E_2^0$ semiq./hydroq.</td>
<td>-495 mv</td>
<td>-419 mv</td>
<td>-399</td>
<td>-371</td>
<td>-410</td>
</tr>
<tr>
<td>Reference</td>
<td>this study</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
</tbody>
</table>

d. Dubourdieu (1972).
of all the flavodoxins. In fact, this potential is the most negative reduction potential of any biologically important redox enzyme determined to date, being at least 30 mv more negative than that of spinach ferredoxin (Buchanan and Arnon 1970). These results agree well with the reduction of the Shethna protein by hydrogen and hydrogenase as determined by Van Lin and Bothe (1972). They noted that the reduction potential must be very negative, because of the small amount of hydroquinone formed upon hydrogen reduction, even more negative than that reported for E. coli flavodoxin (-410 mv) and for phytoflavin (-470 mv).

Calculation of the Association Constants for the Various Flavin Redox Forms with the Shethna Apoprotein

By combining the reduction potentials for the Shethna flavoprotein with the published reduction potentials for free flavins (Draper and Ingraham 1968) and with the previously determined association constant of the apoprotein with oxidized FMN, it was possible to calculate the association constants for the semiquinone and hydroquinone forms of FMN with the Shethna apoprotein. The calculations are based on the fact that the free energy is path-independent. The free energies and association constants were calculated from equations (53) and (54).

\[ \Delta G^\circ = -nF E^\circ \] (53)

\[ K_a = e^{-\Delta G^\circ/RT} \] (54)
These results are shown in the scheme below.

\[
\begin{align*}
F + P & \xrightarrow{K_a = 1.7 \times 10^8 \text{ M}^{-1}} PF \\
\Delta G^0 &= -11.1 \text{ Kcal} \\
E^0 &= -275 \text{ mv} \\
\Delta G^0 &= +6.3 \text{ Kcal}
\end{align*}
\]

\[
\begin{align*}
\text{FH}^+ + P & \xrightarrow{K_a = 5.8 \times 10^{13} \text{ M}^{-1}} PFH^+ \\
\Delta G^0 &= -18.6 \text{ Kcal} \\
E^0 &= -219 \text{ mv} \\
\Delta G^0 &= +5.0 \text{ Kcal}
\end{align*}
\]

\[
\begin{align*}
\text{FJ}^- + P & \xrightarrow{K_a = 1.4 \times 10^9 \text{ M}^{-1}} PFH^- \\
\Delta G^0 &= -12.4 \text{ Kcal}
\end{align*}
\]

It is seen that there is a large increase in the binding of flavin in going from the oxidized ($K_a^F = 1.7 \times 10^8 \text{ M}^{-1}$) to the semiquinone ($K_a^{FH^+} = 5.8 \times 10^{13} \text{ M}^{-1}$) form of FMN which partly reverses in going from the semiquinone to hydroquinone form ($K_a^{FH^-} = 1.4 \times 10^9 \text{ M}^{-1}$).

This same pattern, although not as marked, has been calculated for *P. elsdenii* flavodoxin where $K_a^F = 2.3 \times 10^9$, $K_a^{FH^+} = 2.8 \times 10^{11}$ and $K_a^{FH^-} = 1.1 \times 10^8$ (Mayhew 1971a). The magnitude of this enhancement in the Shethna flavoprotein suggests that there may be extensive rearrangement of the flavin binding site upon reduction to the semiquinone, perhaps due to the fact that charge is generated in the
flavin molecule upon formation of this species. The decrease in affinity upon further reduction could be due to the loss of one charge upon forming FH⁻ or to the fact that fully reduced flavin has a bent conformation (Dudley et al., 1964), or both. It is also interesting that free flavin semiquinones show an enhancement in complexation with indoles over that of oxidized flavins (Draper and Ingraham, 1970).

**Determination of the Kinetics of Reduction of the Shethna Flavoprotein**

The kinetics of the semiquinone to hydroquinone conversion of the Shethna flavoprotein were determined by temperature-jump methods. Equilibrium concentrations of semiquinone and hydroquinone were varied by changing the pH in the presence of excess dithionite (Edmondson and Tollin, 1971, p. 133). Methyl viologen (MV) was used to mediate the reduction.

The mechanism below was shown to be operative in agreement with the results of Edmondson and Tollin (1971, p. 133).

\[
PFH^* + [H^+] \xrightleftharpoons[k_2]{k_1} PFH_2 \xrightleftharpoons[k_4]{k_3} PFH^- + H^+ \tag{55}
\]

Under the conditions of the experiment, only a single relaxation was observed indicating that a steady state existed with respect to the concentration of PFH₂ (Figure 38). Therefore the mechanism can be abbreviated using apparent rate constants as shown below.

\[
PFH^* + MV^* \xrightleftharpoons[k_{11}]{k_1} FP^- + H^+ + MV \tag{56}
\]
Figure 38. Plot of log $\Delta G$ vs. time for the determination of the relaxation time for the Shethna flavoprotein semiquinone/hydroquinone equilibrium.

pH 7.8, 0.1 M triz buffer,
8.8 mole % methyl viologen, $3 \times 10^{-5}$ M protein
where

\[ k^1 = \frac{k_1 k_3}{k_2 + k_3} \]  
\[ k^{11} = \frac{k_2 k_4}{k_2 + k_3} \]  

With the large excess of dithionite (1 mg/ml) and strong buffer concentration (0.1 M), both the reduced methyl viologen [MVH\(^-\)] and pH were effectively buffered such that the relationship between the relaxation time and the apparent rate constants was greatly simplified as shown in equation (59).

\[ \frac{1}{\tau} = k^1 [MVH^-] + k^{11} [H^+] \]  

Therefore, by holding the pH constant, a plot of \( \frac{1}{\tau} \) vs. [MVH\(^-\)] should be linear with a slope of \( k^1 \) and intercept of \( k^{11} [H^+] \) (Figure 39). Similarly, by holding the concentration of reduced methyl viologen constant, a plot of \( \frac{1}{\tau} \) vs. [H\(^+\)] should be linear with a slope of \( k^{11} \) and an intercept of \( k^1 [MVH^-] \) (Figure 40). Both methods yielded linear plots and reasonable agreement in the values of \( k^1 \) and \( k^{11} \), indicating that the assumed mechanism was correct. The values of \( k^1 \) and \( k^{11} \) show that the conversion of hydroquinone to semiquinone protein by methyl viologen is two orders of magnitude faster than the conversion of semiquinone to hydroquinone.

Using average values of \( k^1 (5.8 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}) \) and \( k^{11} (4.8 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}) \) obtained from the two series of experiments, the apparent
Figure 39. Plot of $\frac{1}{T}$ vs. [MVH+] for Shethna flavoprotein semiquinone/hydroquinone equilibrium.

$k_{11} = 8.1 \times 10^4 \text{sec}^{-1}$

$k^{-1} = 3.1 \times 10^5 \text{M}^{-1}\text{sec}^{-1}$
Figure 40. Plot of $\frac{1}{t}$ vs. $[H^+]$ for the Shethna flavoprotein semiquinone/hydroquinone equilibrium.

$k_{11} = 3.5 \times 10^{4} \text{ M}^{-1} \text{ sec}^{-1}$

$k_{1} = 6.5 \times 10^{5} \text{ M}^{-1} \text{ sec}^{-1}$
equilibrium constant for the reduction process was calculated from equation (60) where $K_{\text{app}}$ is equal to the ratio of $k_1/k_{11}$.

$$k_{\text{app}} = \frac{k_1}{k_{11}} = \frac{[\text{PFH}^-][\text{H}^+][\text{MV}]}{[\text{PFH}^\cdot][\text{MV}^\cdot]} = 1.2 \times 10^{-2} \text{ M}$$

Using this equilibrium constant, the potential for the reduction of the flavoprotein by methyl viologen can be calculated ($E^o = -110 \text{ mv}$). Combining this information with the reduction potential for methyl viologen (Clark 1960), the potential for the conversion from the semiquinone to the hydroquinone form of the flavoprotein can also be calculated as illustrated below.

$$\begin{align*}
\text{PFH}^\cdot + \text{MV}^\cdot &\rightleftharpoons [\text{PFH}^-][\text{H}^+][\text{MV}] \\
&= 1.2 \times 10^{-2} \text{ M} \\
&\text{PFH}^- + \text{H}^+ + \text{MV} \\
E^o &= -110 \text{ mv} \\
+ &\text{MV} + [\text{H}^+] \\
&\text{MV}^\cdot \\
E^o &= -440 \text{ mv} \\
\text{PFH}^\cdot + [\text{H}^+] &\rightleftharpoons [\text{PFH}^-][\text{H}^+] \\
&\text{PFH}^- + \text{H}^+ \\
E^o &= -550 \text{ mv}
\end{align*}$$

This reduction potential is in reasonable agreement with that determined by potentiometric titration (-495 mv). This provides further support for the assumed mechanism. It would appear, then, that temperature-jump relaxation provides no indication of any conformational changes accompanying the conversion of semiquinone to fully reduced protein.

A temperature-jump experiment was also performed using the $P$. elsdenii flavodoxin under conditions similar to those involving the Shethna flavoprotein. In order to achieve an equilibrium mixture of the semiquinone and hydroquinone flavodoxin, excess dithionite
(1 mg/ml) together with methyl viologen (20 mole percent) at pH 5.8 was added to the degassed sample (Mayhew, Foust, and Massey 1969). The relaxation behavior (Figure 41) is similar to that observed for the Shethna flavoprotein semiquinone/hydroquinone interconversion. Although a complete concentration analysis of the relaxation behavior was not performed, it is reasonable to assume that the mechanism of the interconversion is the same as that of the Shethna flavoprotein.

It would also be of interest to determine the kinetics upon going from the oxidized flavoprotein to the semiquinone form. However, this equilibrium could not be maintained as above with a simple manipulation of pH using excess dithionite. Attempts were made to achieve an equilibrium by using a less than stoichiometric amount of dithionite. However, in order to bring the kinetics into an accessible time range, a twofold molar excess of methyl viologen over protein had to be used. In the presence of this excess viologen, the sample became extremely sensitive to oxidation by the slow leakage of oxygen into the temperature-jump cell. Therefore it proved impossible to maintain the oxidized/semiquinone equilibrium and the kinetics could not be determined. It should be noted that the kinetics of the semiquinone/hydroquinone equilibrium were not effected by oxygen, due to the presence of the large excess of dithionite.
Figure 41. Relaxation trace and semilog plot for the *P. elsdenii* flavodoxin semiquinone/hydroquinone equilibrium.

pH 5.8; 0.1 M PO₄ buffer; 10 mole % methyl viologen; 3 x 10⁻⁵ M protein.
Although the results of this study have been discussed in some detail already, it is worthwhile to reiterate and add further comment on some of the main features.

**Free Flavin Reduction Mechanisms**

It has been shown that the kinetic mechanisms associated with partially reduced flavin solutions are consistent with earlier proposals; that is, a complex of oxidized and fully reduced flavin is a precursor to the flavin semiquinone (Gibson et al. 1962, Beinert 1960, Fox and Tollin 1966). However, the differences in the rates of radical disproportionation as determined by flash photolysis and temperature-jump experiments have been resolved in this study. It was found that the rates differ due to the formation (at high flavin concentrations) of a complex between oxidized and reduced flavin that contains considerable radical dimer character. The complex is best represented as an equilibrium mixture of a charge transfer complex and a radical dimer with about equal contribution from each. It has been shown previously that many crystalline complexes of flavins with aromatics (Tollin 1968) are also paramagnetic and that this paramagnetism is due to the trapping of unpaired electrons at imperfections within the crystal. However, the maximum unpaired spin which is produced in these cases corresponds to about 1 mole percent of the complex. Therefore
radical dimer character has not been ascribed to these complexes. In light of the high spin concentration (30-50%) of the paramagnetic species isolated in the present study, these complexes represent the first neutral and anion flavin radical forms that have been isolated as relatively stable solids.

**Flavin-protein Interactions**

Through the use of several flavin analogs, the importance of the ribityl phosphate side chain and N-5 ring position in the flavin-protein interaction has been explored. It has been suggested on the basis of the multiple relaxation behavior in the binding of phosphorylated analogs that the phosphate group induces a conformational change in the protein subsequent to the initial binding process. This is further substantiated by the large net entropy change accompanying the binding of FMN and the activation entropy change found for the dissociation step. In addition, the slower leaving rates for the phosphorylated analogs are indicative of a phosphate-induced closure of the flavin binding site. The results of this study are consistent with earlier work in which Edmondson and Tollin (1971, p. 113) using CD spectroscopy have shown that there is a reversible change in protein secondary and tertiary structure upon binding of FMN to the Shethna apoprotein. More recently, D'Anna and Tollin (1972) have demonstrated this same effect for the flavodoxins from *C. pasteurianum*, *P. elsdenii*, *D. vulgaris*, and *R. rubrum*. Inasmuch as the *P. elsdenii* flavodoxin shows relaxation behavior similar to that of the Shethna flavoprotein it may be reasonable to expect similar binding mechanisms for all of
the flavodoxins. Additional experiments along this line would be of interest.

It has also been shown that the ribityl side chain does not contribute to the mechanism of binding inasmuch as riboflavin, lumiflavin, and deazariboflavin all bind by a simple second order process. In addition, the N-5 position does not influence the mechanism of binding inasmuch as deazaFMN and FMN show similar relaxation behavior. The ribityl side chain and N-5 position do however influence the rates of binding. This is most probably the result of steric factors in the binding of riboflavin and lumiflavin, and the result of the loss of a small degree of side chain - N-5 cooperativity for the deaza derivatives. Although the N-5 position only influences the kinetics of flavin binding quantitatively, it has been shown in this study that this position is crucial for the stabilization of the flavoprotein semiquinone. Further support for this has been given by Edmondson and Tollin (1971, p. 133) who showed that the increased rate of semiquinone oxidation (for the Shethna flavoprotein) by oxygen with increased pH was most likely due to the state of protonation at the N-5 position. Mayhew et al. (1969) have observed similar behavior for the P. elsdonii flavodoxin.

It has also been shown by Edmondson and Tollin (1971, p. 133) that the phosphate group of the ribityl side chain is essential for stable semiquinone formation. This is again consistent with a phosphate-induced conformational change (this study) as a result of which
the access of reducing agent to the flavin ring may be restricted and
protonation of the position stabilized.

Model experiments involving the kinetics of complexation of
flavins with aromatics revealed little information that would be of use
in the interpretation of which groups in the protein are responsible
for flavin binding. About all that can be said is that, if complexation
of flavins with tryptophan and tyrosine is important in the binding of
flavins to proteins, this is probably not a rate-determining interac-
tion. However, one should keep in mind that complexation of flavins
with aromatics may also be important in the metabolic role of flavo-
proteins. A number of flavoenzymes have been shown to develop broad,
long wavelength, absorption bands upon the addition of certain aro-
matic compounds that have been attributed to complex formation (Massey
and Palmer 1962). It is quite possible that flavoprotein-substrate-
inhibitor interactions may involve complexation as a component. The
rapid rate of complexation found in the present study would be signifi-
cant in terms of efficient catalysis and inhibition. In addition, the
complexation of physiologically active indoles (such as serotonin) with
flavoproteins may be responsible for the biological action of these
materials. Temperature-jump kinetic experiments involving flavoprotein
complexation would be of great interest here and a productive area for
future work.

Flavoprotein Reduction Properties

Analysis of the temperature-jump relaxation behavior of the
Shethna flavoprotein semiquinone/hydroquinone equilibria revealed only
a single relaxation that is indicative of the absence of conformational changes accompanying interconversion. This is consistent with earlier studies (Edmondson and Tollin 1971, p. 113) in which it was found that the far U.V. CD spectra of the oxidized, semiquinone, and hydroquinone forms of the flavoprotein were essentially identical. Therefore, the large enhancement in the binding energy of semiquinone flavin over that of hydroquinone flavin must not result from a conformational change in the protein, or if there is a conformational change, it must be relatively small and highly localized.

It is also interesting that the *P. elsdenii* flavodoxin showed similar relaxation behavior to that of the Shethna flavoprotein. This indicated that the mechanism of reduction is probably the same for both proteins. Additional experiments involving other flavodoxins would be of interest.

The reduction potentials for the Shethna flavoprotein (\(E_1^O = +50\) mv and \(E_2^O = -495\) mv) are extremely interesting in that they are consistent with the previously determined redox behavior of this enzyme [e.g.: (a) semiquinone stability to oxygen (Hinkson and Bulen 1967, Edmondson and Tollin 1971, p. 133); (b) ability to catalyze nitrogen reduction (Benemann et al. 1969); (c) ability to replace ferredoxin in NADP\(^+\)-photoreduction (Van Lin and Bothe 1972); (d) oxygen reactivity of the hydroquinone (Hinkson and Bulen 1967, Edmondson and Tollin 1971, p. 133)]. The fact that the hydroquinone form of the Shethna flavoprotein is the most powerful reducing enzyme yet determined may aid in the elucidation of its metabolic role. Because the
Shetna flavoprotein is naturally occurring (i.e., not induced through iron deprivation) the second reduction potential cannot be compared with a ferredoxin that it replaces (inasmuch as *Azotobacter* simultaneously produces a ferredoxin), as with most of the other flavodoxins. Therefore, a search for a metabolic substrate is necessary in order to understand the biological significance of such a negative reduction potential.
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