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Flavine–Protein Interactions in Flavoenzymes. Temperature-Jump and Stopped-Flow Studies of Flavine Analog Binding to the Apoprotein of *Azotobacter* Flavodoxin†

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ABSTRACT: Equilibrium binding constants, kinetic parameters, and thermodynamic parameters have been determined for the binding of several flavine analogs to the apoprotein of *Azotobacter* flavodoxin (Shethna flavoprotein). The results indicate that the ribityl phosphate side chain and the N-5 position of the isoalloxazine ring act cooperatively during the rate-limiting step of the binding process. Kinetic measurements using temperature-jump and stopped-flow methods provide strong evidence for a phosphate-induced protein conformational change. The values for the individual rate constants and activation entropies are interpreted in terms of the detailed processes which occur during coenzyme binding. Preliminary studies using *Peptostreptococcus elsdenii* flavodoxin suggest that the mechanism of flavine mononucleotide binding is similar to that of the *Azotobacter* protein.

Studies of flavine–protein interactions have been facilitated by the fact that in most flavoenzymes the flavine is not covalently bound to the protein and may be resolved by treatment with acid (Warburg and Christian, 1938; Hinkson, 1968) or KBr dialysis (Massey and Curti, 1966). The resolved apoprotein may then be recombined with modified flavines and the enzymatic activity (when possible), redox behavior, and spectral properties compared to those of the native holoenzyme. In this way, the positions on the isolloxazine ring and ribityl side chain that influence the above properties can be determined.

We have measured the kinetics and thermodynamics of binding of several flavine analogs to the Shethna apoprotein (*Azotobacter* apoflavodoxin).[1] This flavoprotein is particularly useful for this type of study because of its high stability, low molecular weight, and possession of only one flavine binding site. The flavine mononucleotide (FMN) cofactor is easily resolved and can be subsequently recombined with essentially complete renaturation as evidenced by circular dichroism (CD) and redox properties (Edmondson and Tollin, 1971a,b). The apoprotein will efficiently bind other flavine 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, 1971b; Edmondson *et al.*, 1972). It has also been observed that the presence of the 5′-phosphate group increases the flavine–protein association constant for *Azotobacter* flavodoxin (Hinkson, 1968; Edmondson and Tollin, 1971c). To date, all of the studies concerning flavine–protein interactions have used static methods such as absorption, fluorescence, and CD spectroscopy of analog, holo-, and apoflavoproteins, titration methods, equilibrium analog-binding studies, and simple kinetic measurements of rate limiting steps in analog binding. Some of this work has suggested that the phosphate group of FMN induces a protein rearrangement that facilitates the binding process (Edmondson and Tollin, 1971b; D'Anna and Tollin, 1972). However, there is no kinetic evidence to support this mechanistic proposal.

Relaxation methods (Eigen, 1954) provide a powerful tool for the study of rapid biological reactions that are mechanistically complicated. For each independent step in a reaction

† From the Department of Chemistry, University of Arizona, Tucson, Arizona 85721. *Received July 10, 1972.* Supported in part by a grant from the National Institutes of Health (1R01-AM15057) and taken from a dissertation submitted by B. G. B. in partial fulfillment of requirements for a Ph.D. degree, University of Arizona, 1972.

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[1] Although 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, more recent studies by Van Lin and Bothe (1972) have demonstrated that this flavoprotein can indeed substitute for ferredoxin and thus can be classified as a flavodoxin.

mechanism there will be a separate relaxation process. Therefore, a knowledge of the individual relaxation times and their concentration behavior will allow the determination of the kinetic parameters which characterize a 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. In the present work, we have used the temperature-jump method to investigate the mechanism of binding of flavine analogs to *Azotobacter* apoflavodoxin.

Experimental Section

Materials

Flavine Analogs. Riboflavine (RBF)[2] and FMN were obtained from Calbiochem, Los Angeles, Calif. Riboflavine was used without further purification. FMN was purified by DEAE-cellulose column chromatography at pH 7.0. 5-Deazariboflavine was obtained from Dr. C. C. Cheng, Midwest Research Institute, Kansas City, Mo. 5-DeazaFMN was prepared by the method of Flexser and Farkas (1952) from 5-deazaRbf and was purified by the DEAE-cellulose column chromatography. Lumiflavin was previously synthesized using the method of Guzzo and Tollin (1963). Riboflavine sulfate ($Rbf \cdot SO_4$) 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 K_2HPO_4 at pH 7.0 as the eluent. Concentrations were determined spectrophotometrically using published values for the molar extinctions (Beinert, 1960; Yagi, 1971).

Flavoproteins. Azotobacter flavodoxin (Shethna flavoprotein) was isolated from *Azotobacter vinelandii*, 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, Mich. Protein concentrations were determined spectrophotometrically using published extinction coefficients (Hinkson and Bulen, 1967; Mayhew and Massey, 1969). The method of preparation of the apoprotein of *Azotobacter* flavodoxin, using 3% trichloroacetic acid and dithiothreitol, was identical with that described by Edmondson and Tollin (1971c). The concentration of apoprotein was determined spectrophotometrically using a molar extinction of 29,000 $M^{-1}\,cm^{-1}$ at 280 nm.

Other Chemicals. Tryptophan and tyrosine derivatives were obtained from Calbiochem, Los Angeles, Calif. Serotonin was a gift from Dr. A. Picchioni, University of Arizona, Department of Pharmacology.

Methods

A joule heating temperature-jump spectrophotometer was constructed for the measurement of rapid reaction rates. The basic design is similar to that first proposed by Eigen (1954). A Sorensen Model 230-6P-RD high-voltage dc power supply was used to charge a 40-kV low inductance 0.1-μF capacitor (Plastic Capacitor, Inc., Chicago, Ill.). An E. G. & G. Model 14B spark gap and Model TR60 trigger module provided the high-voltage switching circuit.

A General Electric type EKG Quartzline lamp (80 W), driven by a Sorensen Q-Nobatron (QB 18-6) highly stabilized dc power supply, and passed through a Bausch & 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 dc power supply. Both load resistance and capacitance were variable, giving a continuously variable RC filter with a response time from 1 μsec to 100 msec. The output from the detector was fed into a Tektronix Model 533A oscilloscope. Transients were recorded using a C-27 Tektronix oscilloscope camera with Polaroid type 107 film.

The design of the cell which was used in these studies is a conglomerate based upon our own experience with Eigen-type cells (Eigen, 1954) and a cell designed by Czerlinski (1962). It required slightly more than 1 ml of solution to fill. The use of conical quartz lenses as windows greatly enhanced its optical sensitivity and allowed for both absorption and fluorescence detection. The conical lenses proved to be an absolute necessity for the fluorescence detection of kinetic transients in the present work. The lens specifications are the same as that proposed by Czerlinski (1962). Virtually all of the sample was held between the electrodes and heated and therefore there was no reservoir of cooler sample to mix with the heated liquid following a temperature jump. This extended the upper limit of accessible relaxation times to more than thirty seconds before appreciable cooling occurred. The exponential heating time was approximately 11 μsec for a 7°C temperature change.

In addition to the temperature-jump method, a Gibson-Durrum stopped-flow spectrophotometer was also used for the determination of the binding kinetics of several flavine analogs to the apoflavodoxin. This instrument was modified for fluorescence detection by replacing the normal observation cell with a fluorescence cell.

The kinetics of binding of flavine analogs were determined at pH 7.5 in mixed Tris–phosphate buffer (0.1 M in each) containing 0.1 M KNO_3. Samples were prepared from stock solutions using an equimolar concentration of flavine and protein. For studies of FMN binding, the native holoprotein was used. Most of the kinetic runs were carried out at 10°. For the binding of lumiflavine, riboflavine, and FMN, kinetics were also determined at 24°.

The association constants of flavine analogs to *Azotobacter* apoflavodoxin were determined at pH 7.0 in 0.025 M phosphate buffer. Fluorescence quenching titration of the flavine by apoprotein was used (Edmondson and Tollin, 1971c). Gibbs free energies were calculated at 24° for all analogs. Enthalpies and entropies of binding were also determined for riboflavine, lumiflavine, and FMN by performing the titrations at 10°.

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 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 synthe-

[2] Abbreviations used are: Rbf, riboflavine; $Rbf \cdot SO_4$, riboflavine sulfate.

TABLE I: Equilibrium Binding Constants of Flavine Analogs to *Azotobacter* Apoflavodoxin.

Flavine	K_a (24°)	Cor Coef	K_a (10°)	Cor Coef	$\Delta H°$ (kcal/mol)	$\Delta G°_{24°}$ (kcal/mol)	$\Delta S°_{24°}$ (cal/(deg mol))
FMN	1.7×10^8 [a]	0.96	2.4×10^8	0.98	−4.1	−11.1	23.6
Riboflavine	1.8×10^6 [a]	0.99	3.0×10^6	0.97	−6.1	−8.5	8.1
Lumiflavine	2.2×10^5 [b]	0.96	3.7×10^5	0.92	−6.2	−7.2	3.4
Rbf·SO_4	6.3×10^7	0.99				−10.6	
DeazaFMN	4.0×10^7 [c]	0.99				−10.3	
DeazaRbf	4.2×10^5	0.99				−8.0	

[a] These values are in good agreement with those obtained by Edmondson and Tollin (1971c). [b] Edmondson and Tollin (1971c). [c] Edmondson *et al.* (1972).

sized 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.

All straight-line plots were constructed using a least-squares fitting procedure.

Results and Discussion

Association Constants and Equilibrium Thermodynamics. The six flavine derivatives that were used in the determination of association constants (K_a) are represented in Figure 1. Riboflavine and lumiflavine differ from FMN by the absence of the phosphate and ribityl phosphate groups, respectively. Riboflavine 5′-sulfate differs from FMN by the replacement of phosphate by sulfate. In the deaza derivatives, the N-5 ring nitrogen has been replaced by carbon.

The results are given in Table I. As was observed previously (Edmondson and Tollin, 1971c), the phosphorylated side chain significantly enhances binding, suggesting a strong charge–charge interaction between flavine and protein (compare FMN and deazaFMN with riboflavine, lumiflavine, and deazariboflavine). Riboflavine sulfate binds only slightly less strongly than does FMN, showing that the negatively charged sulfate substitutes well for phosphate. 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 (compare FMN with deazaFMN and riboflavine with deazariboflavine).

Inspection of the thermodynamic constants reveals a larger free energy and smaller enthalpy of binding for FMN than for either riboflavine or lumiflavine. This is reflected in a much larger entropy term for FMN. This large entropy change 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 Bindings. The rate law for the binding of flavine analogs to apoprotein should be simple second order, in the absence of any subsequent first-order conformational steps. If such is the case, in a temperature-jump experiment only one relaxation would be seen. The relaxation time for this mechanism

$$F + P \underset{k_1}{\overset{k_2}{\rightleftharpoons}} FP \qquad (1)$$

should obey

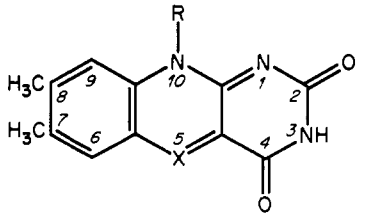
$$\frac{1}{\tau} = k_2([\bar{F}] + [\bar{P}]) + k_1 \qquad (2)$$

where k_2 and k_1 are the forward and back rate constants, and $[\bar{F}]$ and $[\bar{P}]$ are the new equilibrium concentrations of free flavine and apoprotein. Therefore, 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.

If two relaxations are observed for the binding of a flavine analog to apoprotein, it is reasonable to assume that these reflect a bimolecular step in which flavine (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

$$F + P \underset{k_1}{\overset{k_2}{\rightleftharpoons}} X \underset{k_4}{\overset{k_3}{\rightleftharpoons}} FP \qquad (3)$$

Linearization of the independent rate equations for the above



Compound	R=	X=
FMN	$CH_2(CHOH)_3CH_2OPO_3$	N
Riboflavine	$CH_2(CHOH)_3CH_2OH$	N
Lumiflavine	CH_3	N
Deaza-FMN	$CH_2(CHOH)_3CH_2OPO_3$	C
Deaza-RBF	$CH_2(CHOH)_3CH_2OH$	C
RBF-SO_4	$CH_2(CHOH)_3CH_2OSO_3$	N

FIGURE 1: Structures of flavine derivatives used in this study.

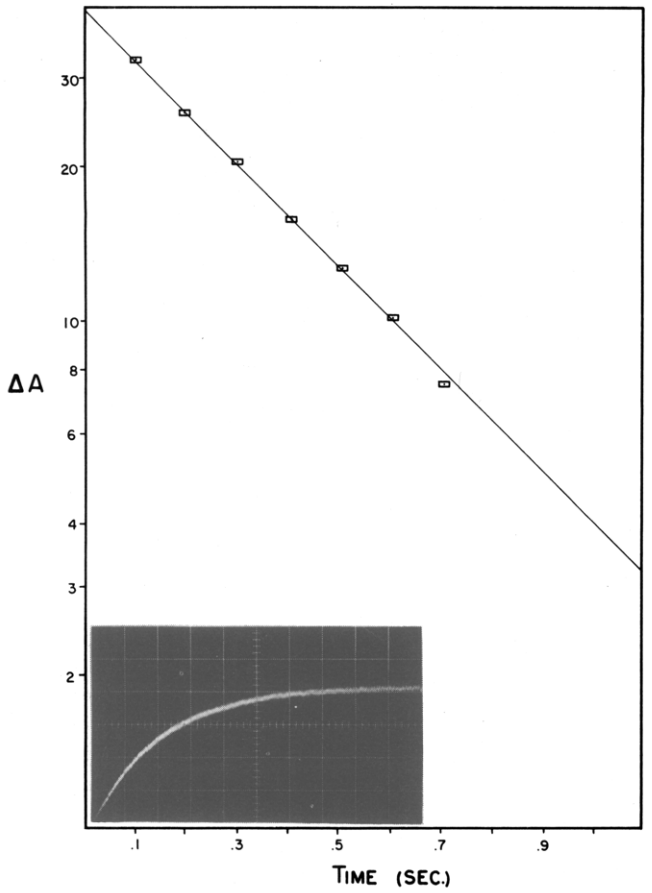


FIGURE 2: Plot of log ΔA *vs.* time for the determination of the relaxation time for the binding of riboflavine (2×10^{-5} M) to *Azotobacter* apoflavodoxin (2×10^{-5} M). Inset shows experimental trace (fluorescence increases along vertical axis, 0.2 sec/division); 0.1 M phosphate buffer (pH 7.5)–0.1 M KNO_3.

mechanism leads to two expressions involving the relaxation times (eq 4 and 5)

$$\frac{1}{\tau_1} + \frac{1}{\tau_2} = k_2([\bar{F}] + [\bar{P}]) + k_1 + k_3 + k_4 \qquad (4)$$

$$\frac{1}{\tau_1} \times \frac{1}{\tau_2} = \frac{k_3 K_{12}([\bar{F}] + [\bar{P}])}{1 + K_{12}([\bar{F}] + [\bar{P}])} + k_4 \qquad (5)$$

The two relaxation times can be determined as a function of free flavine and apoprotein concentrations by analog com-

TABLE II: Rate Constants at 24° for the Binding of Flavine Analogs to Azotobacter Apoflavodoxin.

$$F + P \underset{k_1}{\overset{k_2}{\rightleftharpoons}} FP$$

Analog	k_2 (M^{-1} sec^{-1})	k_1 (sec^{-1})
FMN	$2.0 \pm 0.4 \times 10^5$	$1.2 \pm 0.2 \times 10^{-3}$
Riboflavine	$8.5 \pm 0.5 \times 10^5$	0.50 ± 0.03
Lumiflavine	4.0×10^7	1.8×10^2
RbF-SO_4	5.6×10^5	8.8×10^{-2}
5-DeazaFMN	$4.1 \pm 0.2 \times 10^4$	$1.0 \pm 0.05 \times 10^{-3}$
5-DeazaRbf	3.6×10^5	0.80

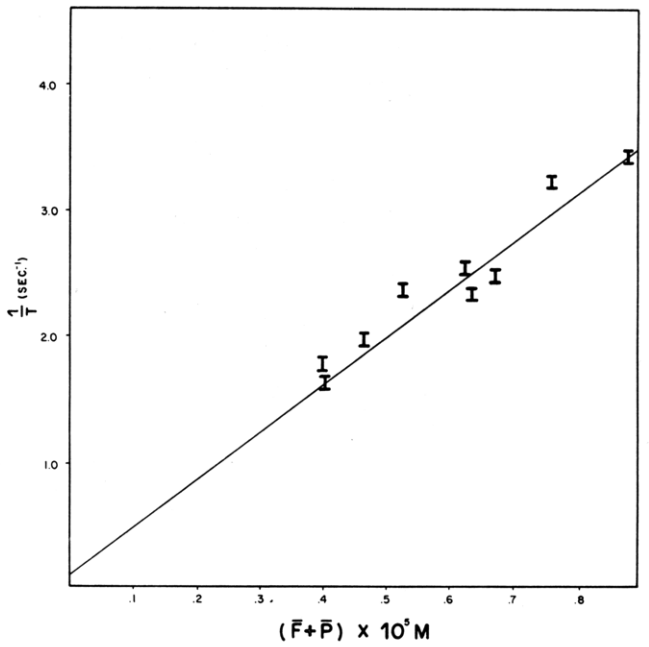


FIGURE 3: Plot of $1/\tau$ *vs.* $[\bar{F}] + [\bar{P}]$ for riboflavine binding to *Azotobacter* apoflavodoxin.

puter fitting of the experimental data, as described above. It is possible, from a knowledge of $1/\tau_1$ and $1/\tau_2$ 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 (riboflavine, lumiflavine, and deazariboflavine) only a single relaxation was observed. This is verified by the linearity of a plot of log ΔS (signal) *vs.* time. The relaxation times are determined from the slope of such a plot. A typical relaxation trace (riboflavine binding) along with its log plot is shown in Figure 2. Plots of $1/\tau$ *vs.* $([\bar{F}] + [\bar{P}])$ for lumiflavine and riboflavine are shown in Figures 3 and 4 and the calculated rate constants are given in Table II. Kinetic constants for deazari-

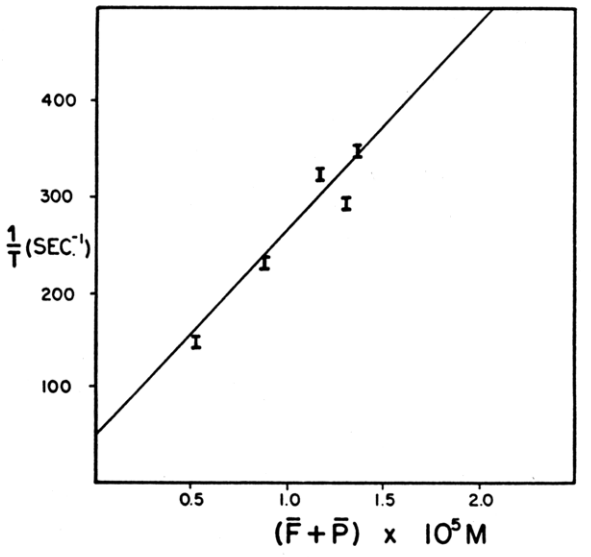


FIGURE 4: Plot of $1/\tau$ *vs.* $[\bar{F}] + [\bar{P}]$ for lumiflavin binding to *Azotobacter* apoflavodoxin.

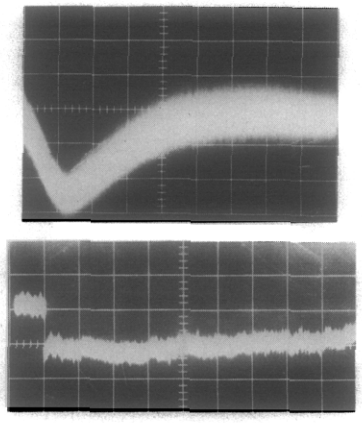


FIGURE 5: Relaxation trace for the binding of FMN (10^{-4} M) to *Azotobacter* apoflavodoxin (10^{-4} M) (upper curve) together with FMN cooling time blank (lower curve); 5 sec/division. The rapid downward deflection (fluorescence decrease) in the lower trace is due to the fact that the FMN fluorescence yield is lower at the higher temperature; this quenching occurs within the heating time of the instrument; pH 7.5, 0.025 M phosphate buffer.

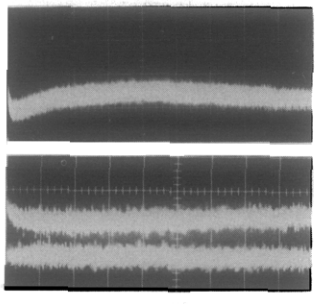


FIGURE 6: Relaxation traces for the binding of 5-deazaFMN to *Azotobacter* apoflavodoxin at pH 7.5. The same sample (6×10^{-5} M in both analog and protein) was used for both traces. The sample also contained 0.05 M phosphate buffer and 0.1 M KNO_3. Upper trace, 5 sec/division; lower trace, 0.2 sec/division.

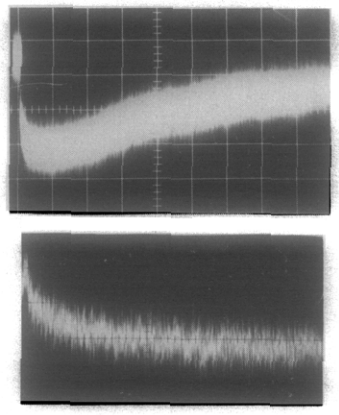


FIGURE 7: Relaxation traces for binding of FMN to *P. elsdenii* flavodoxin apoprotein at pH 6.8. The same sample (8×10^{-5} M in both flavine and apoprotein) was used for both traces. The sample also contained 0.025 M phosphate buffer and 0.1 M KNO_3. Upper trace, 5 sec/division; lower trace, 0.2 sec/division.

boflavine were obtained by determining $1/\tau$ at only a few concentrations and then calculating the rate constants using the previously determined association constant (Table I).

Using mixing experiments, Edmondson and Tollin (1971c) have shown that the kinetics of binding of FMN to *Azotobacter* apoflavodoxin are simple second order. Stopped-flow experiments have verified this behavior. However, this does not preclude a multiple step process, if the rate limiting step is second order and a faster first-order step follows. Temperature-jump experiments show that the kinetics are indeed not simple second order. Two separate relaxations were observed, suggesting a mechanism of the type indicated in eq 3.

In Figure 5 is shown a typical relaxation trace obtained for the binding of FMN. Also shown in Figure 5 is a cooling time blank 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.[2] Similar results were obtained using the riboflavine plus apoprotein system or phenol red to determine cooling times. A comparison of the FMN relaxation trace to that shown in Figure 2 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 subsequent 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 suggest the following. 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 flavine and apoprotein (X), in which the flavine is not well buried within the protein, would exhibit less flavine fluorescence quenching than one in which the flavine 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 ($X \rightleftharpoons FP$) is endothermic. The latter would be consistent with a protein conformational change requiring significant disruption of secondary and tertiary structure. Edmondson and Tollin (1971c) and D'Anna and Tollin (1972) have suggested that there is a change in protein structure upon the binding of FMN, based upon a comparison of the far-uv CD spectra of the apo and holo forms of several flavodoxins.

Similar relaxation behavior to that found with FMN is observed for 5-deazaFMN (Figure 6), 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 cooperativity between the phosphate group and this position in the binding process. A similar conclusion is arrived at on the basis of stopped-flow experiments (*cf.* below and Edmondson *et al.*, 1972). Temperature-jump experiments with riboflavine sulfate were not successful due to partial hydrolysis of the sulfate group to form riboflavine during the course of the experiment.

It is interesting that temperature-jump experiments using *P. elsdenii* flavodoxin showed the same relaxation behavior as was found with *Azotobacter* flavodoxin (Figure 7). Although a concentration dependence of the relaxation times was not performed, the general shape of the curve is quite

[2] There is a small amount of cooling during the time course of the experiment. This proceeds at a rate which is negligible in terms of the relaxation analysis.

similar and thus the mechanism of FMN binding is probably identical.

In Table II, the forward and reverse rate constants for the binding of the flavine analogs to the apoprotein are summarized. It should be noted that k_1 for FMN is an apparent rate constant, where

$$k_{1,\text{app}} = \frac{k_1 k_4}{k_3} \tag{6}$$

This relation is obtained by dividing k_2 by the overall equilibrium constant. In addition, the rate constants given[3] for FMN, 5-deazaFMN, and riboflavine sulfate were determined by the stopped-flow method, while the temperature-jump method was used for riboflavine, lumiflavine, and 5-deazariboflavine.

Inspection of Table II 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 flavine–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 crystallographic work by Dr. M. Ludwig (1971)[4] has shown that in a Clostridial flavodoxin the flavine (FMN) is largely buried in a cleft in the protein. A similar situation also exists for the *Desulfovibrio vulgaris* flavodoxin (Jensen, 1972).[4] Edmondson and Tollin (1971b) have reached similar conclusions about the binding site in *Azotobacter* flavodoxin 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 lumiflavine leaves the binding site much more rapidly than does riboflavine. 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 riboflavine 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 to 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 protein conformational rearrangement proceeds faster with the deaza analog than with FMN). It is possible that the preferred conformation of the coenzyme for binding is a folded one in which the N-5 nitrogen and the phosphate group present themselves simultaneously to the apoprotein. This would imply that the flavine binding site within the apoprotein has a complementary relationship with respect to protein functional groups involved in binding. In addition, the faster rate at which riboflavine 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 was possible to evaluate all four rate constants for both steps. Three experimental traces and the analog computer curves which best fit the data,[5] along with the plots of log ΔS *vs.* t for the two computer-derived exponentials, are shown in Figures 8–10. Because of the poor signal-to-noise ratio in these experiments, it was not possible to vary the concentration over a wide range. In Figure 11, a plot of $1/\tau_1 + 1/\tau_2$ *vs.* $[\bar{F}] + [\bar{P}]$ is given from which the rate constants k_2 and the sum $k_1 + k_3 + k_4$ are determined. As $1/\tau_1$ is constant, and $1/\tau_2$ at zero $[\bar{F}] + [\bar{P}]$ is determined by the above plot, k_4 can be calculated from eq 5. 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 computed. Since all four rate constants are now known, the individual equilibrium constants $K_{12} = k_2/k_1$ and $K_{34} = k_3/k_4$ are calculated. The results are given in Table III. In spite of the small range over which the concentration was varied, all kinetic constants are believed to be at least within an order of magnitude or better of the true rate constants.

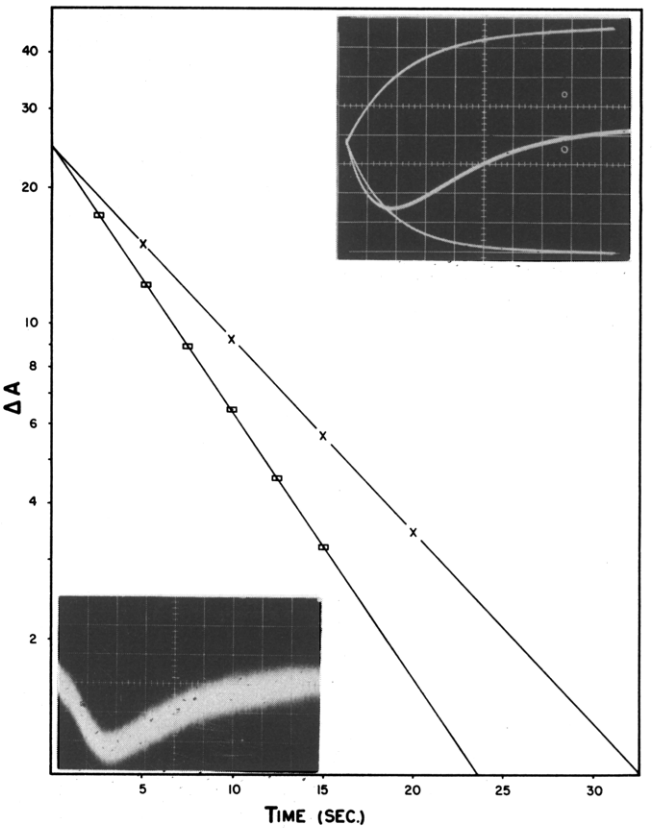


FIGURE 8: Plot of log ΔA *vs.* time for the determination of $1/\tau_1$ and $1/\tau_2$ for *Azotobacter* flavodoxin (10^{-4} M) at pH 7.5. Experimental (lower) and analog computer resolution (upper) traces superimposed; 5 sec/division. (×) $1/\tau_1 = 0.137$ sec^{-1}. (⊡) $1/\tau_2 = 0.100$ sec^{-1}.

The initial association constant (K_{12}) is seen to be comparable to the association constant for riboflavine, 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

[3] These experiments allow a true second-order constant to be determined, inasmuch as the initial binding step is rate limiting and $k_1 \ll k_3$ (*cf.* Table III).

[4] Personal communication.

[5] 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.

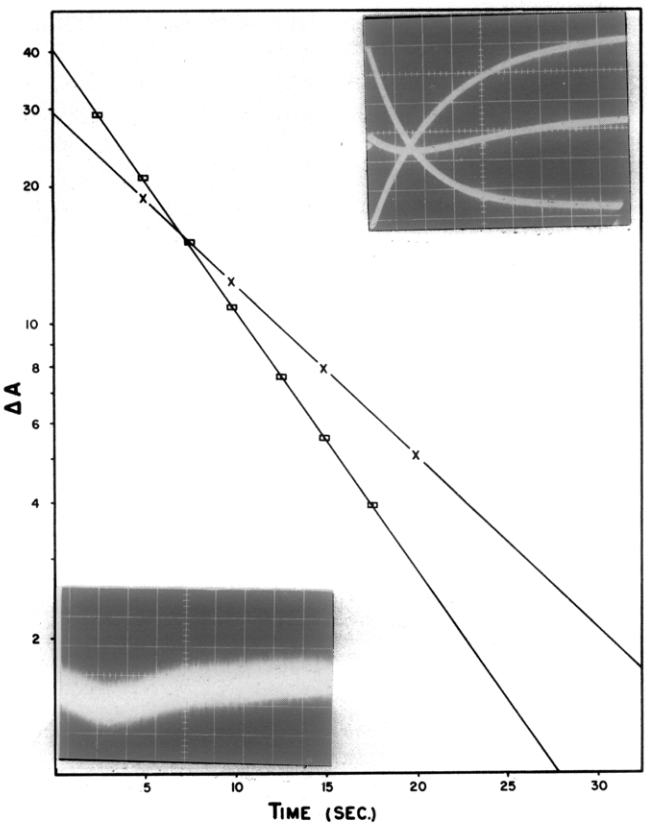


FIGURE 9: Plot of log ΔA *vs.* time for the determination of $1/\tau_1$ and $1/\tau_2$ for *Azotobacter* flavodoxin (7.6×10^{-5} M) at pH 7.5. Experimental (lower) and analog computer resolution (upper) traces superimposed; 5 sec/division. (×) $1/\tau_1 = 0.137$ sec^{-1}. (⊡) $1/\tau_2 = 0.090$ sec^{-1}.

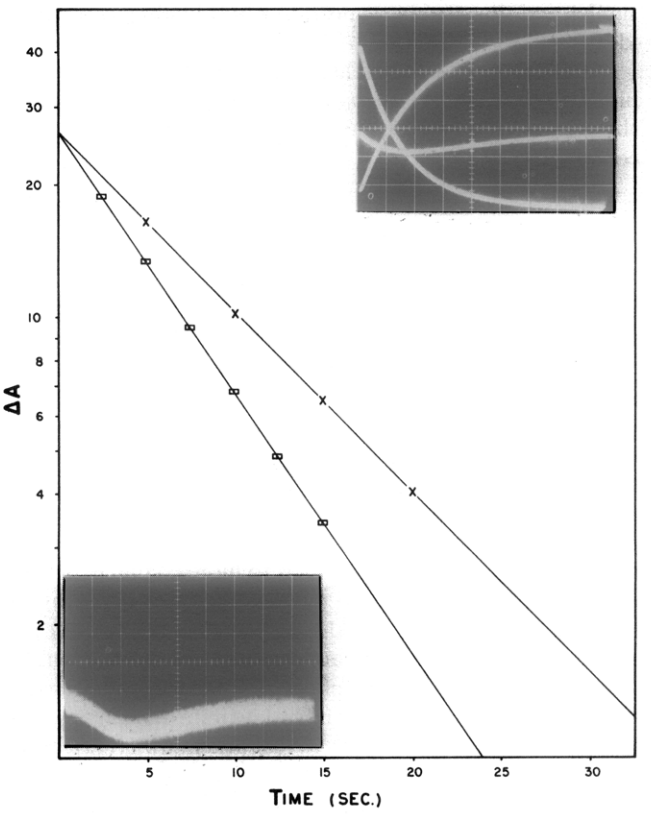


FIGURE 10: Plot of log ΔA *vs.* time for the determination of $1/\tau_1$ and $1/\tau_2$ for *Azotobacter* flavodoxin (5.2×10^{-5} M) at pH 7.5. Experimental (lower) and analog computer resolution (upper) traces superimposed; 5 sec/division. (×) $1/\tau_1 = 0.137$ sec^{-1}. (⊡) $1/\tau_2 = 0.080$ sec^{-1}.

increase in the association constant of FMN over that of riboflavine. 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 still another step in the binding process which is fast and corresponds to a fluorescence increase. The duration and magnitude of this relaxation made it difficult to carry out an 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 riboflavine. (If a small amount of bound riboflavine was present, this would result in a fast fluoroscence increase upon heating, as observed.)

Determination of Activation Parameters. For the binding of FMN, riboflavine, and lumiflavine, kinetics were determined at two temperatures in order to obtain an estimate of the Arrhenius energy and entropies of activation. The results

TABLE III: Rate and Equilibrium Constants for the Binding of FMN to *Azotobacter* Apoflavodoxin (Constants Defined by eq 3).

Rate Constants (10°)	Equil Constants (10°)
$k_2 = 5.3 \times 10^4$ M^{-1} sec^{-1}	
	$K_{12} = 6.5 \times 10^6$ M^{-1}
$k_1 = 8.1 \times 10^{-3}$ sec^{-1}	
$k_3 = 1.6 \times 10^{-1}$ sec^{-1}	
	$K_{34} = 37$
$k_4 = 4.2 \times 10^{-3}$ sec^{-1}	
pH 7.0 in 0.025 M phosphate buffer	

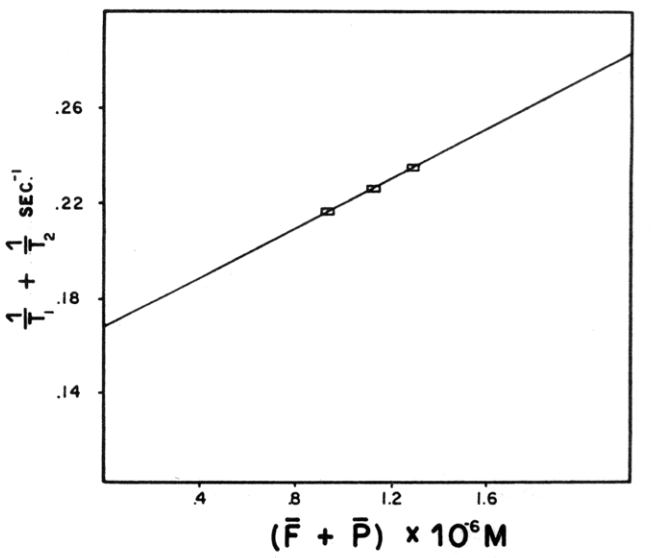


FIGURE 11: Plot of $1/\tau_1 + 1/\tau_2$ *vs.* $[\bar{F}] + [\bar{P}]$ for the determination of the rate constants for the binding of FMN to *Azotobacter* apoflavodoxin (pH 7.5, 10°).

TABLE IV: Arrhenius Energy and Entropy of Activation as Determined by Kinetics at Two Temperatures.

	Forward Rate Constants F + P ⟶ FP				Reverse Rate Constants FP ⟶ F + P			
	k_2 (24°), M^{-1} sec^{-1}	k_2 (10°), M^{-1} sec^{-1}	E_A, kcal/mol	ΔS^*_2 (24°), cal/(deg mol)	k_1 (24°), sec^{-1}	k_1 (10°), sec^{-1}	E_A, kcal/mol	ΔS^*_1 (24°), cal/(deg mol)
FMN	2.0×10^5	5.3×10^4	15.8	17.0	1.2×10^{-3}	2.1×10^{-4}	20.7	−3.9
Riboflavine	8.9×10^5	3.9×10^5	9.3	−2.1	0.50	0.13	16.0	−7.7
Lumiflavine	4.0×10^7	2.2×10^7	7.1	−1.7	180	59	13.3	−5.4

are given in Table IV. For FMN, the back rate constant is again the apparent constant as defined previously.

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 riboflavine and lumiflavine. Although the interpretation of activation parameters for large molecules is difficult, it is probably safe to say that the differences are due mainly to the protein–phosphate and solvent–phosphate interactions which occur as a consequence of the burial of the phosphate group within the binding site. In the activated complex for the initial rate-determining 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 release of the coenzyme, 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 riboflavine and lumiflavine would reflect mainly solvation effects, inasmuch as a protein conformational rearrangement does not occur. The much smaller entropy changes in the association steps for riboflavine and lumiflavine (as compared to 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.*, lumiflavine and riboflavine > FMN. Such a mechanism is also consistent with the somewhat larger rate constant observed for riboflavine sulfate (Table II), 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 riboflavine and lumiflavine is probably due mainly to steric effects.

Kinetics of Complex Formation of Flavines with Aromatic Compounds. It is well established that flavines will form complexes with many aromatic compounds (Tollin, 1968). Of particular interest is the complexation with tryptophan and tyrosine (Isenberg and Szent-Györgi, 1958) since such complex formation may be responsible in part for binding of flavines in flavoproteins (Tollin, 1968; Draper and Ingraham, 1970). Also of interest is the binding of other indoles to flavines, 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-methylindole. However, in all experiments, even at concentrations of 10^{-5} M, 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 V). The calculated rate constants are more than two orders of magnitude larger than those observed for FMN binding to apoprotein (*cf.* Table II and Edmondson and Tollin, 1971c), and it thus appears that even though flavine complexation with tryptophan and tyrosine may be important in the flavine–protein interaction, it certainly is not a rate-determining interaction for the binding process.

Conclusions

Through the use of several flavine analogs, the importance of the ribityl phosphate side chain and N-5 ring position in the flavine–protein interaction has been explored. It has been suggested on the basis of the complex relaxation behavior which occurs during 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 flavine binding site. The results of this study are consistent with earlier work (Edmondson and Tollin, 1971c) using CD spectroscopy in which it was shown that there is a reversible change in protein secondary and tertiary structure upon binding of FMN to *Azotobacter* apoflavodoxin. More recently, D'Anna and Tollin (1972) have demonstrated this same effect for the flavodoxins from *Clostridium pasteurianum*, *P. elsdenii*, *Desulfovibrio vulgaris*, and *Rhodospirillum rubrum*. Inasmuch as the *P. elsdenii*

TABLE V: Minimum Rate Constants for FMN Complexation.

Aromatic Donor	Bimolecular Rate Constant (M^{-1} sec^{-1})	Monomolecular Rate Constant (sec^{-1})	Ass. Constant
Tryptophan	$>1 \times 10^7$	$>1 \times 10^5$	90[a]
Tyrosine	$>1 \times 10^7$	$>1 \times 10^5$	66[a]
Serotonin	$>4 \times 10^7$	$>1 \times 10^5$	400[a]
3-Methylindole	$>2 \times 10^7$	$>1 \times 10^5$	50[b]
Quinoxaline	$>1 \times 10^7$	$>1 \times 10^5$	100[c]
2,3-Naphthalenediol	$>2 \times 10^7$	$>1 \times 10^5$	240[d]

[a] Draper and Ingraham (1970). [b] Tollin (1968). [c] Estimated. [d] Fleischman and Tollin (1965).

flavodoxin shows relaxation behavior similar to that of *Azotobacter* flavodoxin 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 riboflavine, lumiflavine, and deazariboflavine 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 riboflavine and lumiflavine, 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 flavine binding quantitatively, it has been shown previously (Edmondson *et al.*, 1972) that this position is crucial for the stabilization of the flavoprotein semiquinone. Further support for this has been given by Edmondson and Tollin (1971b) who showed that the increased rate of semiquinone oxidation (for *Azotobacter* flavodoxin) 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. elsdenii* flavodoxin.

It has also been shown by Edmondson and Tollin (1971b) 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 as a result of which the access of redox agents to the flavine ring may be restricted and protonation of the N-5 position stabilized.

An interesting aspect of the present studies is the fact that two structurally related compounds (Rbf and FMN) are able to bind strongly to the same protein, but only one of them can induce a conformational rearrangement. It is not unlikely that this type of protein–ligand interaction pattern is involved in a wide variety of inhibitor and antagonist effects observed with enzymes, drug receptor sites, etc.

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