The Binding of Flavin Derivatives to the Riboflavin-binding Protein of Egg White

A KINETIC AND THERMODYNAMIC STUDY

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A method is described for the preparation and isolation of a riboflavin-binding protein in gram quantity. The product is found to be homogeneous in size and in binding character but slightly heterogeneous in net charge; it consists of a single polypeptide chain of about 30,000 daltons when examined by SDS gel electrophoresis. An affinity chromatography method is described by which flavin analogs and impurities can be separated on G-50 Sephadex.

Aporiboflavin-binding protein complexes a variety of flavin analogs with >95% quenching of flavin fluorescence. The weight of aporiboflavin-binding protein required to quench the fluorescence of 1 mol of riboflavin equals the molecular weight of the protein, demonstrating that 1 mol of aporiboflavin-binding protein contains 1 mol of riboflavin binding sites. The other flavin analogs also bind with 1:1 stoichiometry.

Van't Hoff analyses for binding to aporiboflavin-binding protein of more than 20 flavin analogs at pH 7.0 are found to be linear over the temperature range from 10-40 °C. Equilibrium binding energies lie in the ranges: ΔG, from -7 to -15 kcal/mol, ΔH, from -13 to -20 kcal/mol, and ΔS, from -3 to -34 e.u./mol. Several sites on the flavin are identified as important to binding; however, no one site dominates the interaction.

The kinetics of flavin binding to aporiboflavin-binding protein is examined by stopped flow fluorimetry. The quenching of the flavin fluorescence is accurately described by a unique second order rate constant under a variety of conditions, demonstrating that the quenching process monitors the association reaction. Arrhenius analyses are linear over the temperature range from 10 °C to 40 °C and yield activation energies for flavin analog binding which lie in the ranges: ΔG, from 3.5 to 5.5 kcal/mol, ΔH, from 4.5 to 8 kcal/mol, and ΔS, from -2 to +12 e.u./mol.

The relaxation kinetics for the dissociation of several analog-apoprotein complexes is examined at several temperatures. For most complexes, the binding process is found to be adequately described by a single step equilibrium, allowing the dissociation rate constants for the complexes to be calculated from the equilibrium and association rate constants. Because the variation in the magnitude of the association rate constant is very much less than the variation in the equilibrium constants, it is concluded that the dissociation process determines the strength of the interaction.

The interaction between flavin derivatives and proteins depends predominantly on noncovalent interaction between the two components, although there are now well established examples of protein-flavin complexes in which the linkage between the two components is by covalent coupling of the isoalloxazine ring to the protein.

To date, the most widely used approach for characterizing the flavin-protein interaction has been the study of the dependence of the equilibrium constant for the flavin-protein complex on chemical modification of either or both of the two components, flavin and protein (2, 3). Although the strategy has yielded important insights in general, the method suffers because most flavo-apoproteins show a high specificity for their related flavin, and, thus, the extent to which the flavin can be modified is quite limited.

The riboflavin-binding protein from egg white appears to have the physiological role of providing vitamin B2 for the developing embryo. It is unusual in that it will bind a large number of flavin derivatives, and it is, therefore, a particularly favorable system by which to characterize the factors controlling the flavin-protein interaction, especially as the resultant flavoprotein complex shows little or none of the fluorescence characteristic of free flavin.

In this paper, we describe a rapid and convenient procedure for the purification of the holoprotein of the riboflavin-binding protein from egg white together with a simple technique for quantitative removal of bound flavin. Some elementary properties of the isolated protein are reported, and our detailed results on the thermodynamics and kinetics of binding of a large number of flavin analogs to the egg white apoprotein are described.

MATERIALS AND METHODS

The supplemental material contains a description of the "Materials and Methods," the preparation of flavin-saturated riboflavin-binding protein and its conversion to the apoprotein, a presentation of the physical properties of the isolated protein and its conversion to the apoprotein, and additional references are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-2913, cite authors, and include a check or money order for $5.20 per set of photocopies. Full sized photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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properties of the isolated holoprotein including the stoichiometry of flavin binding, and a discussion of procedures and considerations pertinent to the quantitative measurement of the strength of flavin binding (also included are Figs. 1 to 3 and Table II).

**RESULTS**

*Equilibrium Studies on the Binding of Flavins by Apo-riboflavin-binding Protein*—The results of a fluorescence titration are shown in Fig. 4, together with the behavior predicted for an ideal binding system using the average value of the dissociation constant ($K_d$). The data at low saturation of flavin exhibit the greatest uncertainty because the absolute magnitude of the error in free flavin is relatively constant for all data points, and the free and bound protein concentrations are determined by difference measurements involving $[P]_b$, $[P]_s$, and free flavin. Thus, the relative error in the two protein species is considerably larger for the first addition of apoprotein than for subsequent additions and this results in an increased uncertainty in the determinations involving the first addition.

Evaluating the $K_d$ values for flavin binding at each point between 10 and 90% saturation served a useful purpose. The consistency of the individual values gave some qualitative information concerning the homogeneity of the flavin analog under consideration. With most analogs, relatively little variation in $K_d$ occurred over the range from 10 to 90% of the initial fluorescence quenched. However, minor variations were often observed, with $K_d$ values calculated near the end of the titration 5 to 30% higher than the $K_d$ value obtained at the beginning. We routinely used the average as providing the most reliable value.

Constant values for $K_d$ were taken to indicate that the system of flavin plus protein was substantially homogeneous with respect to binding. A significantly greater variation in $K_d$ over the 10 to 90% quenching range observed for a given flavin analog was interpreted as suggesting inhomogeneity in the analog (e.g. the 3,4-dihydroriboflavin sample (Table II) which appeared to be contaminated with riboflavin).

The Effects of pH and Ionic Composition on the Equilibrium Constant for Riboflavin Binding to the Apoprotein—The results of fluorescence quenching titrations in 0.1 M acetate, phosphate, and Tris buffers in the range from pH 5 to pH 9 at 25 °C are shown in Fig. 5. The affinity of the protein for flavin is nearly constant between pH 6 and pH 9 and independent of the nature of the buffers tested. The binding constant was also determined at other pH values using CD measurements, making the assumption that the CD intensity of the flavoprotein and of the free reactants is independent of pH. The fluorescence quenching and the CD data are (not shown) in good agreement.

To determine whether pH affected the temperature dependence of the equilibrium constant, limited van’t Hoff analyses were performed under various conditions of pH and buffering ions. No systematic deviations were observed.

Some experiments were performed which examined the effect of ions on the binding of riboflavin to apoprotein. Insignificant variations in $K_d$ (25 °C) were found using fluorescence quenching determinations in 0.01 M or 0.1 M potassium phosphate, pH 7.0, in the presence and absence of 0.1 M KCl. The presence of 1 M KBr or 2 M NaN$_2$SO$_4$ caused no change in the observed CD spectrum of native holoprotein in 0.1 M Tris-Cl, pH 7.5. This established an upper limit of 7 × 10$^{-9}$ M for $K_d$ under these conditions.

The observation that flavins can be dissociated from flavoproteins by dialysis against KBr solutions at low pH values (4, 5) prompted a study of riboflavin binding as a function of

### Table II

<table>
<thead>
<tr>
<th>Flavin</th>
<th>$K_d$ (25 °C)</th>
<th>$\Delta G$ (25 °C)*</th>
<th>$\Delta H$</th>
<th>$\Delta S$ (25 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>1.3 × 10$^{-9}$</td>
<td>-14.7 ± 0.2</td>
<td>-20.3 ± 0.2</td>
<td>-19 ± 1</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>4.7 × 10$^{-9}$</td>
<td>-12.4 ± 0.2</td>
<td>-17.3 ± 0.1</td>
<td>-17 ± 1</td>
</tr>
<tr>
<td>Lumichrome</td>
<td>9.3 × 10$^{-9}$</td>
<td>-12.0 ± 0.2</td>
<td>-17.3 ± 0.2</td>
<td>-18 ± 1</td>
</tr>
<tr>
<td>10-Hydroxyethyl flavin</td>
<td>2.9 × 10$^{-8}$</td>
<td>-12.7 ± 0.1</td>
<td>-18.7 ± 0.3</td>
<td>-20 ± 1</td>
</tr>
<tr>
<td>Tetraacetylriboflavin</td>
<td>1.9 × 10$^{-9}$</td>
<td>-10.4 ± 0.3</td>
<td>-20.1 ± 0.5</td>
<td>-34 ± 2</td>
</tr>
<tr>
<td>FMN</td>
<td>1.4 × 10$^{-6}$</td>
<td>-10.4 ± 0.3</td>
<td>-20.1 ± 0.5</td>
<td>-33 ± 3</td>
</tr>
<tr>
<td>FAD</td>
<td>&gt;1.4 × 10$^{-5}$</td>
<td>&gt;8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methyl lumiflavin</td>
<td>5.1 × 10$^{-9}$</td>
<td>-12.4 ± 0.2</td>
<td>-14.6 ± 0.2</td>
<td>-7 ± 1</td>
</tr>
<tr>
<td>3-Benzyl lumiflavin</td>
<td>9.5 × 10$^{-9}$</td>
<td>-13.3 ± 0.2</td>
<td>-17.2 ± 1.0</td>
<td>-13 ± 3</td>
</tr>
<tr>
<td>Lumiflavin 3-acetate</td>
<td>4.5 × 10$^{-7}$</td>
<td>-11.1 ± 0.1</td>
<td>-14.8 ± 0.4</td>
<td>-13 ± 2</td>
</tr>
<tr>
<td>3,4-Dihydroriboflavin</td>
<td>2.8 × 10$^{-8}$</td>
<td>-12.7 ± 0.3</td>
<td>-16.2 ± 0.3</td>
<td>-12 ± 1</td>
</tr>
<tr>
<td>Deam riboflavin</td>
<td>5.9 × 10$^{-9}$</td>
<td>-13.7 ± 0.3</td>
<td>-18.9 ± 0.4</td>
<td>-18 ± 2</td>
</tr>
<tr>
<td>Isolamiflavin</td>
<td>8.2 × 10$^{-8}$</td>
<td>-12.1 ± 0.1</td>
<td>-15.4 ± 0.2</td>
<td>-11 ± 1</td>
</tr>
<tr>
<td>7,10-Dimethylisalloxazine</td>
<td>4.8 × 10$^{-7}$</td>
<td>-11.0 ± 0.1</td>
<td>-14.4 ± 0.2</td>
<td>-11 ± 1</td>
</tr>
<tr>
<td>10-Methylalloxazine</td>
<td>3.7 × 10$^{-7}$</td>
<td>-11.2 ± 0.1</td>
<td>-15.0 ± 0.1</td>
<td>-13 ± 1</td>
</tr>
<tr>
<td>7-Chlorolumiflavin</td>
<td>2.0 × 10$^{-8}$</td>
<td>-12.9 ± 0.1</td>
<td>-18.2 ± 0.1</td>
<td>-18 ± 2</td>
</tr>
<tr>
<td>8-Chlorolumiflavin</td>
<td>8.1 × 10$^{-8}$</td>
<td>-12.1 ± 0.2</td>
<td>-16.2 ± 0.1</td>
<td>-14 ± 1</td>
</tr>
<tr>
<td>7,8-Dichlorolumiflavin</td>
<td>4.2 × 10$^{-8}$</td>
<td>-12.5 ± 0.2</td>
<td>-15.9 ± 0.4</td>
<td>-12 ± 2</td>
</tr>
<tr>
<td>3-Methyl,8a-morpholinolumiflavin</td>
<td>5.0 × 10$^{-8}$</td>
<td>-9.7 ± 0.3</td>
<td>-17.5 ± 1.0</td>
<td>-26 ± 3</td>
</tr>
<tr>
<td>8a-Histidyl lumiflavin</td>
<td>&gt;2 × 10$^{-4}$</td>
<td>&gt;7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3-Dimethylalloxazine</td>
<td>4.0 × 10$^{-8}$</td>
<td>-12.5 ± 0.3</td>
<td>-13.4 ± 1.1</td>
<td>-3 ± 4</td>
</tr>
<tr>
<td>1,3,7,8-Tetramethylalloxazine</td>
<td>4.2 × 10$^{-8}$</td>
<td>-12.5 ± 0.2</td>
<td>-17.1 ± 0.5</td>
<td>-16 ± 3</td>
</tr>
<tr>
<td>1,3,7,8-Tetramethyl 4-methoxyalloxazine</td>
<td>2.4 × 10$^{-7}$</td>
<td>-11.4 ± 0.2</td>
<td>-15.1 ± 0.8</td>
<td>-12 ± 3</td>
</tr>
</tbody>
</table>

*Both $\Delta G$ (25 °C) were converted to the corresponding unitary quantities by correction for the cratic contribution (19); $\Delta S$, (25 °C) is 8 e.u./mol more positive than $\Delta S$, (25 °C), and $\Delta G$, (25 °C) is 2.4 kcal/mol more negative than $\Delta G$, (25 °C).*
Flavin Binding to Riboflavin-binding Protein

**FIG. 5.** Variables affecting the affinity of riboflavin for the binding protein. Left, pH dependence of the dissociation constant. Dissociation constants \( K_d \) were obtained either by fluorescence quenching (\( \times \)) or from CD measurements (\( \circ \)). For the fluorescence experiments, the buffers used were 0.1 M sodium acetate (pH 4.8 to 6.0), 0.1 M potassium phosphate (pH 6.0–8.0), 0.1 M Tris-Cl (pH 7.0–9.0), and 0.1 M glycine HCl (pH 10.4). For the CD experiments, the solvent was 0.1 M KCl and the pH of the reaction mixture was adjusted to the indicated value using either sodium hydroxide or hydrochloric acid. Right, effect of anionic strength and anions on the dissociation constant. \( \chi \), Fluorescence quenching data in 0.01 M potassium phosphate or 0.1 M phosphate + 0.1 M NaCl. CD data taken in 0.1 M Tris-Cl, pH 7.0 (\( \times \)), 0.1 M sodium acetate, pH 4.1 (\( \circ \)), or 0.1 M sodium acetate, pH 3.8 (\( \triangle \)). In each case, potassium bromide, sodium chloride, or ammonium sulfate was present, as shown, to produce the desired ionic strength.

**FIG. 6.** Van't Hoff plots for the binding of five flavin analogs by aporiboflavin-binding protein in 0.01 M potassium phosphate, pH 7.0.

KBr concentration. Although KBr apparently had little effect on the binding at pH 7.5 (see above), increasing concentrations of KBr dramatically decreases the \( K_d \) for riboflavin binding at more acid pH values (Fig. 5). NaCl has a similar but less dramatic effect at pH 3.8. These observations are consistent with the results reported by Mayhew (5) for flavodoxins. The relatively greater effect of Br\(^-\) and Cl\(^-\) is consistent with the order and approximate magnitude of the effect observed for Old Yellow Enzyme by Theorell and Nygaard (6).

Van't Hoff Analyses for Analog Binding—Fluorescence quenching titrations for more than 20 flavin analogs were carried out over the temperature range 10–40 °C. The data generally consisted of several titrations at five or more temperatures spaced over the temperature range. No indication of curvature was observed for any of the analogs (Fig. 6). \( \Delta H \) for each analog was calculated from the least squares fit to the slope of the plot of the logarithm of the (average) \( K_d \) against \( T^{-1} \). Error limits on each \( \Delta H \) value were established from the 95% confidence interval on the slope of the “best fit” line; \( \Delta G_{298} \) (25 °C) was calculated from the interpolated value of \( K_d \) at 25 °C. Each error limit in \( \Delta G_{298} \) (25 °C) represents the relative standard deviation of the differences between the observed \( K_d \) at all temperatures and the “best fit” line. \( \Delta S_{298} \) (25 °C) was then evaluated from \( \Delta H \) and \( \Delta G_{298} \) (25 °C), and the error limit in \( \Delta S \) was set equal to the larger of the error limits in \( \Delta H \) and \( \Delta G_{298} \) (25 °C). Table II summarizes the results of the equilibrium studies on the binding of flavin analogs by aporiboflavin-binding protein.

Kinetic Studies on the Binding of Flavins by Aporiboflavin-binding Protein—Equal concentrations of riboflavin and apoprotein were mixed in the stopped flow fluorimeter and the ensuing decrease in fluorescence followed with time. When the initial concentration of each reactant was \( 10^{-5} \) M, plots of the reciprocal of fluorescence intensity versus time were linear, indicative of an irreversible second order reaction. However, when the initial concentration of reactants was lowered to \( 10^{-7} \) M, curvature was observed in the second order

---

**TABLE III**

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>After mixing</th>
<th>( 10^3 K_d ) pseudo-first order analysis</th>
<th>( 10^3 ) ( k ) reversible analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 ( \times ) [apoprotein]</td>
<td>1.7 ( \times ) [riboflavin]</td>
<td>( 10^3 [\text{M}^{-1} \cdot s^{-1}] )</td>
<td>( 10^3 [\text{s}^{-1}] )</td>
</tr>
<tr>
<td>( 10^{-7} )</td>
<td>( 10^{-7} )</td>
<td>4.1 ± 0.2</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>( 10^{-6} )</td>
<td>( 10^{-6} )</td>
<td>3.8 ± 0.3</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>( 10^{-5} )</td>
<td>( 10^{-5} )</td>
<td>4.2 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>( 10^{-4} )</td>
<td>( 10^{-5} )</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>( 10^{-3} )</td>
<td>( 10^{-6} )</td>
<td>3.8 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>( 10^{-2} )</td>
<td>( 10^{-6} )</td>
<td>3.8 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
</tbody>
</table>

Averages: 4.0 ± 0.3 4.0 ± 0.2
plot, and the rate constants obtained under the latter conditions were always higher than those obtained at the higher concentrations. This nonlinearity suggested that the reaction was not irreversible, and that the binding reaction was proceeding to an equilibrium fluorescence level. For a reversible second order reaction, the expression for the rate constant is (10):

$$k_t = \frac{2.303 \log ([P] + [P] - [PF] \cdot [PF])}{[F] \cdot [P]}$$  \hspace{1cm} (1)$$

where \([PF]\) is the concentration of the complex at time \(t\) and \([PF]_e\) is the equilibrium concentration. This equation yields a value for the association rate constant at all reagent concentrations. A complementary series of kinetic experiments was conducted using concentrations of apoprotein in excess.

Table III summarizes the results of both kinds of experiments. The observed rate varied by 10,000-fold for the group of experiments conducted under equimolar concentrations and by 10-fold for the group of experiments under conditions of excess apoprotein, yet the values of the rate constant describing the quenching process are seen to be quite similar within each group and between the two groups. Even though the actual quenching process may occur at a step(s) after the initial bimolecular event, fluorescence quenching occurs sufficiently fast that the second order step is rate limiting. Quenching of fluorescence, therefore, monitors formation of the encounter complex and can be used to determine \(k_t\) of Equation 1. Furthermore, the reversible analysis gives values for the rate constant which are consistent with both the values obtained under pseudo-first order conditions and those values obtained from an irreversible analysis of experiments done at high equimolar reactant concentrations. We conclude that the reversible analysis yields an accurate measure of the value for the second order rate constant.

**Arrhenius Analyses for Flavin Analog Binding to Aporo-**

**Table IV**

<table>
<thead>
<tr>
<th>Flavin analog</th>
<th>(k_t) (25 °C)</th>
<th>(\Delta G^\ddagger) (25 °C)</th>
<th>(\Delta H^\ddagger)</th>
<th>(\Delta S^\ddagger) (25 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>4.8 x 10^7</td>
<td>4.5 ± 0.1</td>
<td>4.8 ± 0.1</td>
<td>11.0 ± 0.3</td>
</tr>
<tr>
<td>Lumichrome</td>
<td>1.50 x 10^6</td>
<td>3.9 ± 0.3</td>
<td>6.3 ± 0.2</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>2.5 x 10^6</td>
<td>3.6 ± 0.5</td>
<td>8.2 ± 0.2</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>10-Hydroxethyl flavin</td>
<td>9.7 x 10^7</td>
<td>4.1 ± 0.3</td>
<td>7.4 ± 0.4</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Tetracycliriboflavin</td>
<td>4.9 x 10^7</td>
<td>4.4 ± 0.1</td>
<td>4.4 ± 0.8</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>FMN^a</td>
<td>8.1 x 10^6</td>
<td>5.4 ± 0.1</td>
<td>5.0 ± 0.6</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>3-Methyl lumiflavin</td>
<td>1.75 x 10^6</td>
<td>3.7 ± 0.2</td>
<td>5.4 ± 0.2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>3-Benzyl lumiflavin</td>
<td>1.65 x 10^6</td>
<td>3.9 ± 0.4</td>
<td>6.1 ± 0.7</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Lumiflavin 3-acetate</td>
<td>3.8 x 10^7</td>
<td>4.6 ± 0.4</td>
<td>7.2 ± 0.5</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>3,4-Dihydroriboflavin^b</td>
<td>3.3 x 10^7</td>
<td>4.7 ± 0.4</td>
<td>7.7 ± 0.4</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Deaza riboflavin</td>
<td>8.9 x 10^7</td>
<td>4.2 ± 0.3</td>
<td>5.9 ± 0.4</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Isolumiflavin</td>
<td>1.65 x 10^8</td>
<td>3.8 ± 0.3</td>
<td>7.7 ± 0.3</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>7,10-Dimethylsialo-</td>
<td>1.15 x 10^8</td>
<td>4.0 ± 0.2</td>
<td>6.2 ± 0.7</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>10-Methylsialoxazine</td>
<td>1.20 x 10^8</td>
<td>4.0 ± 0.4</td>
<td>6.0 ± 0.3</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>7-Chlorolumiflavin</td>
<td>1.65 x 10^8</td>
<td>3.8 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>8-Chlorolumiflavin</td>
<td>1.45 x 10^8</td>
<td>3.9 ± 0.2</td>
<td>6.8 ± 0.3</td>
<td>9 ± 1</td>
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<tr>
<td>7,8-Dichlorolumiflavin</td>
<td>1.50 x 10^8</td>
<td>3.9 ± 0.2</td>
<td>6.9 ± 0.2</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>3-Methyl,8a-morpholin-</td>
<td>5 x 10^7</td>
<td>5 ± 1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1,3-Dimethylaxazine</td>
<td>1.5 x 10^8</td>
<td>3.9 ± 0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a The Arrhenius plots in these cases consisted of data points at only two temperatures.

^b The values for 3,4-dihydroriboflavin are subject to some uncertainty as a result of the apparent contamination of this analog with riboflavin.

**Fig. 7.** Representative Arrhenius plots for the association of riboflavin and some analogs to the binding protein.

**Fig. 8.** Representation examples of relaxation traces for dilution experiments of mixtures of apoprotein with eight norlumiflavin (top), lumiflavin (middle), and FMN (bottom). Each trace consists of the average of 3–12 experiments which had been accumulated in a computer. To the right of each trace is the derived semilogarithmic plot.
at 40 °C were slightly below the line extrapolated from lower temperatures (e.g. riboflavin and lumiflavin, Fig. 7). The Arrhenius energy \( E_a \) was obtained from a linear regression of log \( k_1 \) on \( T^{-1} \) and \( \Delta H^\ddagger \) calculated as \( E_a/RT \). The free energy of activation was calculated from \( \Delta G^\ddagger (\text{kilocalories}) = 17.4 - 1.36 k_1 \).

The standard deviation of the differences between the observed \( k_1 \) values for a given analog and the \( k_1 \) values on the “best fit” line was used to determine the error limits on \( \log k_1 \) on \( T^{-1} \) and \( \Delta H^\ddagger \) calculated as \( E_a/RT \). The free energy of activation was calculated from:

\[
\Delta G^\ddagger (\text{kilocalories}) = 17.4 - 1.36 k_1
\]

The results are summarized in Table IV.

**Relaxation Kinetics**—Relaxation studies were conducted with several analog-apoprotein complexes to establish whether the kinetics could be adequately described by a single exponential and whether the values of \( k_{-1} \) obtained at different temperatures were equal to the product \( k_1K_d \) calculated for the several complexes at the different temperatures.

Relaxation reactions resulting from flavin dissociation can be observed when solutions of holoprotein are rapidly diluted with an equal volume of buffer. The dissociation of flavin leads to an increase in fluorescence which exponentially approaches a new equilibrium value with a characteristic relaxation time, \( \tau \), given by:

\[
\tau^{-1} = k_1([P]_0 + [F]) + k_{-1}
\]

1:1 dilution does not violate the conditions [6F] \( \ll [F] \), necessary to obtain the above equation. Reliable values of \( k_{-1} \) are obtained when \( k_{-1} \gg k_1([P]_0 + [F]) \). This typically requires the use of concentrations of free flavin too dilute to be reliably observed in the stopped flow fluorimeter available. On the other hand, at much higher concentrations of \([P]_0 + [F]\), such that \( k_{-1} \ll k_1([P]_0 + [F]) < 200 \text{ s}^{-1} \), the experimental relaxation traces would have vastly improved signal to noise, but \( k_{-1} \) would not be defined as precisely. Our compromise was to use a series of solutions of flavoprotein which, when diluted, included concentrations near the \( K_d \) value of the complex. Even so, \( k_{-1} \) can only be readily measured when: 1) \( k_{-1} < 100 \text{ s}^{-1} \), 2) \( K_d > 10^{-1} \text{ M} \), and 3) an excitation maximum occurs at wavelengths above 400 nm.

To examine any possible deviation from simple one-step reversible binding, relaxation experiments were conducted using protein complexes of five analogs selected to give a wide range in the values of \( k_{-1} \) and \( K_d \) and chemical modifications of the “flavin” moiety.

For each analog, relaxation studies were performed at two or more temperatures in the range 10–40 °C. Representative examples of traces and the semilogarithmic plots from them are shown in Fig. 8.

Plots of \( \tau^{-1} \) against \([P]_0 + [F]_0 \) for four analog complexes are given in Fig. 9. The agreement between the observed values of \( \tau \) and the values expected for one-step reversible binding is seen to be good with only one minor exception. In the case of FMN (not shown), the relaxation results yield a value for \( k_{-1} \) less than one-half the values of \( k_1K_d \).

The consistency between the observed \( k_{-1} \) values and those calculated from \( k_1K_d \) also cannot be verified for riboflavin because the very small value of \( K_d \) for this analog requires the use of concentrations so dilute that an inferior signal to noise ratio results.

The scatter in the data in some of the plots in Fig. 9 may warrant future reexamination of these relaxation analyses.

![Fig. 9. Relaxation analyses of the dissociation of four flavins from the flavin-apoprotein system at several temperatures as shown. The lines passing through the data are drawn with slope \( (k_1) \) and intercept \( (k_{-1}) \) obtained from the binding and association kinetics data (Tables II, IV, and V) and thus are established independently of the relaxation measurements.](http://www.jbc.org/)

---

**Flavin Binding to Riboflavin-binding Protein**

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Flavin Binding to Riboflavin-binding Protein

These are the values calculated from the data for the equilibrium constants and second order rate constants under the assumption that binding is a one-step reversible reaction.

<table>
<thead>
<tr>
<th>Flavin</th>
<th>( k_1 ) (25 °C)</th>
<th>( \Delta G^\circ,\text{f} ) (25 °C)</th>
<th>( \Delta H^\circ )</th>
<th>( \Delta S^\circ,\text{f} ) (25 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_1 ) kcal/mol</td>
<td>( e.u./mol )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.061</td>
<td>19.2</td>
<td>28.1</td>
<td>30</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>7.0</td>
<td>16.3</td>
<td>23.6</td>
<td>25</td>
</tr>
<tr>
<td>Lamichrome</td>
<td>23</td>
<td>15.6</td>
<td>25</td>
<td>33</td>
</tr>
<tr>
<td>10-Hydroxethyl flavin</td>
<td>2.8</td>
<td>16.8</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>Tetraacytylribi-</td>
<td>92</td>
<td>14.6</td>
<td>24.7</td>
<td>34</td>
</tr>
<tr>
<td>flavin</td>
<td>( \text{FMN}^\text{a,b} )</td>
<td>11</td>
<td>15.8</td>
<td>25.1</td>
</tr>
<tr>
<td>3-Methyl lumiflavin</td>
<td>8.8</td>
<td>16.1</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>3-Benzyl lumiflavin</td>
<td>1.57</td>
<td>17.2</td>
<td>23.3</td>
<td>20</td>
</tr>
<tr>
<td>Lumiflavin 3-acetate</td>
<td>17</td>
<td>15.7</td>
<td>22.0</td>
<td>22</td>
</tr>
<tr>
<td>3,4-Dihydrolubi-</td>
<td>0.9</td>
<td>17.4</td>
<td>23.9</td>
<td>22</td>
</tr>
<tr>
<td>flavin</td>
<td>( \text{Deaza riboflavin} )</td>
<td>0.52</td>
<td>17.9</td>
<td>24.8</td>
</tr>
<tr>
<td>Isolumiflavin</td>
<td>13.6</td>
<td>15.9</td>
<td>23.1</td>
<td>18</td>
</tr>
<tr>
<td>7,10-Dimethylisoo-</td>
<td>55</td>
<td>15.0</td>
<td>20.6</td>
<td>18</td>
</tr>
<tr>
<td>laloxazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-Methylisooal-</td>
<td>44</td>
<td>15.2</td>
<td>21.0</td>
<td>20</td>
</tr>
<tr>
<td>laloxazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-Chlorolumiflavin</td>
<td>3.4</td>
<td>16.7</td>
<td>24.8</td>
<td>18</td>
</tr>
<tr>
<td>8-Chlorolumiflavin</td>
<td>11.8</td>
<td>16.0</td>
<td>22.8</td>
<td>22</td>
</tr>
<tr>
<td>7,8-Dichlorolumiflavin</td>
<td>6.2</td>
<td>16.4</td>
<td>22.8</td>
<td>22</td>
</tr>
<tr>
<td>3-Methyl,8a-morpholoi-</td>
<td>250</td>
<td>14.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nolumiflavin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3-Dimethylallox-</td>
<td>6</td>
<td>16.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The stoichiometry of binding is established to be 1 mol of flavin/mol of protein because 1) the weight of protein required to quench the fluorescence of 1 mol of flavin agrees with the molecular weight of the protein determined by physical methods and 2) the maximum amount of riboflavin-protein complex is formed when the mole fraction of each component is 0.5 (Fig. 3). These methods show that the apoprotein has only one binding site with a high affinity for riboflavin.

The equilibrium constants for the analog-aporiboflavin-binding protein complexes studied range in value from about 10\(^{-8}\) M to greater than 10\(^{-10}\) M, a fairly wide range, but of intermediate values in regard to the binding to other apoflavoproteins to their natural flavin prosthetic groups. For example, flavin mononucleotide binds to apo-Old Yellow Enzyme (24) and to Peptostreptococcus elsendii apoflavodoxin (59) with greater affinity, while flavin adenine dinucleotide binds to apo-d-amino acid oxidase with a \( K_D \) value about 2 \( \times \) 10\(^{-7}\) M (3) and FMN to luciferase with the surprisingly low value of about 10\(^{-3}\)-10\(^{-4}\) M (14), which is at the lower end of the range covered by analog binding to the aporiboflavin-binding protein.

Although the list of equilibrium-binding energies (Table II) contains the results for binding many different flavin analogs, the major conclusion is that small modification in the flavin structure produces changes in the value of \( \Delta G^\circ \), \( \Delta H^\circ \), and \( T \Delta S^\circ \) no greater than a few kilocalories/mol. Thus, none of the altered groups on the flavin nucleus is absolutely essential to the interaction of flavins with this apoprotein. This result is in contrast to the observations for several other flavoproteins that a single interaction can have decisive control over the binding process. Thus, \( P. \) elsdenii apoflavodoxin does not bind strongly to those flavin analogs which have other than a single phosphate group on the 5' hydroxyl (5), while Azotobacter vinelandii apoflavodoxin (Shethna apoprotein) binds flavin mononucleotide, 3-methyl flavin mononucleotide, and riboflavin, but not 3-methyl riboflavin (14), and apo-Old Yellow Enzyme binds flavin mononucleotide with affinity more than a million times greater than it binds riboflavin (2).

Despite the small differences in energy changes (Table II), several correlations can be drawn with regard to the functional groups on the isooxalazine ring which contribute favorably to the interaction of flavins and the apoprotein. Thus, from a comparison of derivatives which differ only by one modification, it appears that side chain ribose hydroxyl groups, the \( C_4 \) carbonyl, \( N_6 \), and the \( C_8 \) methyl, all stabilize the binding interaction by about 1–2 kcal. The presence of an additional methyl group of \( C_2 \) has no effect, while methylation of either \( N_2 \) or \( N_6 \) enhances binding (Table II).

As judged by differences in free energy change on binding, these loci appear favorable to the interaction of flavin with aporiboflavin-binding protein. To the extent one is justified in summing the differences, a total value for the contributions of these individual interactions can be found. By this method, almost one-half (6 to 7 kcal/mol) of the free energy change on binding of riboflavin (\( \Delta G^\circ = -14.7 \) kcal/mol) is accounted for by the interactions with the functional groups at positions 4, 5, 8, and 10. The total change in free energy, enthalpy, or entropy which accompanies flavin binding appears largely to be composed of small contributions resulting from interactions.
Flavin Binding to Riboflavin-binding Protein

at many positions of the flavin molecule, a result of some importance with regard to biological specificity. A similar pattern of behavior is to be found in the additional analogs studied by Walsh et al. (15) and by Choi and McCormick (16). These investigators also noticed a preference for neutral over anionic species, implying an overall hydrophobic character of the binding domain and documented the effects of substitutions on the ribityl side chain.

However, the validity of the above bookkeeping method is somewhat in question as seen by comparison of the results for lumiflavin with 10-methylisalloxazine and 1,3-dimethylalloxazine with 1,3,7,8-tetramethylalloxazine. The structural difference between these two pairs is the same, yet the difference in free energy changes for the binding of the analogs within the pairs differs by about 1 kcal/mol.

A similar contradiction can be found in the data of Rhodes et al. (8) who compared the binding of riboflavin with 3-methyl riboflavin and lumiflavin with 3-methyl lumiflavin to this protein. Their data imply the difference between riboflavin and 3-methyl riboflavin is greater than that observed between lumiflavin and 3-methyl lumiflavin. Thus, small differences observed in free energy changes may not, in fact, be summable. Future studies should examine this point in more detail.

The greatest differences in free energy change are seen to occur for the binding of those analogs to which relatively large, bulky substituents are attached (Table II). It is important to note, however, that no modification of the flavin structure prevented binding. Apparently the binding site does not place severe steric limitations on the size of the flavin. The results for tetraacetylriboflavin and for FMN and FAD give an indication of a limitation in the volume available in the protein region around the flavin side chain (although the negative charge on the phosphate groups in these analogs probably contributes unfavorably to the interaction). A steric limitation may also exist in the region around position 8, as indicated by the more positive change in free energy of binding for 3-methyl, 8a-morpholinolumiflavin and 8a-histidyl lumiflavin. (Note, however, that the latter is a zwitterion which may contribute to its less favorable binding.)

It is interesting that for those "bulky" analogs for which binding data are available, the observed decrease in free energy results from an unfavorable entropic contribution to the interaction.

Some regions of the binding site appear more accommodating to "bulky" residues attached to the flavin and indicate a lower degree of steric restriction. Thus, a benzylic residue at position 3 actually favors the interaction slightly. Although the binding of 3-benzyl lumiflavin is entropically less favorable than the binding of 3-methyl lumiflavin (a consistent but less dramatic example of the concurrence of "bulk" with an unfavorable ΔS value), a more favorable enthalpy change results in the slightly more favorable free energy change.

From a comparison of the equilibrium energies for many pairs of analogs, it is often noted that two analogs have the same ΔG° value as the result of considerably different ΔH and ΔS° values. This is an example of entropy-enthalpy compensation (17). In order to have strict entropy-activation enthalpy plots for the association of flavin analogs to the apoprotein; B, activation entropy-activation enthalpy plots for the association of flavin analogs; C, correlation of the binding constant and dissociation rate constant. The numbers present in each panel represent different flavin analogs; each number is centered at the coordinates appropriate to its respective analog. The flavins represented are riboflavin (1), tetraacetyliroflavin (2), FMN (3), deaza riboflavin (4), 10 hydroxyethyl flavin (5), 8-chlorolumiflavin (6), lumichrome (7), lumiflavin (8), 1,3,7,8-tetramethylalloxazine (9), 3-benzyl lumiflavin (10), 3-methyl, 8a-morpholinolumiflavin (11), 7-chlorolumiflavin (12), 3,4-dihydroriboflavin (13), 7,8-dichlorolumiflavin (14), isolumiflavin (15), 1,3,7,8-tetramethyl 4-methoxyalloxazine (16), 10 methylisalloxazine (17), 3-O-acetyl lumiflavin (18), 8-norlumiflavin (19), and 3-methyl lumiflavin (20).

pH values in a manner very similar to the pH dependence of specific flavin-binding capacity described by Farrell et al. (9). An increase in Kd value is also seen at pH values above 9, a region not previously studied. Increasing the ionic strength of the flavoprotein solution produces a significant increase in Kd value at low pH values, but apparently not at neutral pH values.

The quenching of flavin fluorescence by apoprotein monitors a kinetically fast binding process which is strictly second
order for all of the analogs studied. This is demonstrated by the agreement among the association rate values for riboflavin binding under widely different relative concentrations of flavin and apoprotein. It is also demonstrated for those analogs which have association rate constants too fast to be studied under pseudo-first order conditions by the adherence at all temperatures to a single step, reversible, second order binding process. The values of the second order rate constants found for the binding of flavin analogs range from $8 \times 10^6$ to about $3 \times 10^8$ M$^{-1}$ s$^{-1}$. These values are quite large and approach the largest observed for the binding of prosthetic groups to their apoproteins or for the binding of substrates to enzymes.

Both the presence of a ribose moiety on position N(10) and the presence of a negative charge on the analog decrease the association rate constant severalfold. The latter observation is consistent with the effects of electrostatic repulsion since the protein bears a net negative charge at pH 7.0. Other modifications in the flavin structure have relatively small effect on the value of the second order rate constant, even the presence of a morpholin function at position 8. These observations are in agreement with the equilibrium and CD binding data to indicate that this site is readily available for interaction and is probably close to or on the surface of the molecule.

For the most part, the activation energies for the association rate vary only slightly from one flavin analog to the next (Table II). The only activation energies which are significantly different from the others are for the binding of FMN and tetraacetylriboflavin. For these analogs, the decrease in enthalpy of activation is almost entirely compensated by a decrease in the entropy of activation. Most of the flavin analogs bind with values for $\Delta G^\ddagger$, $\Delta H^\ddagger$, and $TADS$ of 7, 12, and $-5$ kcal/mol, respectively. These values are also significantly different from the results for analog binding to apo-Old Yellow Enzyme. The compensation plot for the activation enthalpies and entropies of flavin analog binding to the aporiboflavin-binding protein is shown in Fig. 10B. One might expect a higher degree of strict compensation for the activation energies than for the equilibrium energies because the differences in the activation free energies (Table III) are much smaller in absolute magnitude than the differences in the free energy changes (Table II). However, the relative differences are comparable in both cases and, as a result, the compensation plot for the activation energies also shows scatter in the data points. Nevertheless, a trend toward compensation is also evident for the activation enthalpies and entropies.

The relaxation studies on the binding of several flavin analogs to aporiboflavin-binding protein demonstrate that the binding process for many analogs can be adequately described by the single step equilibrium (Equation 1). For a variety of analog-protein complexes at several temperatures, a rapid change in the concentration of the complex induces a relaxation process which is described by a single exponential having a value expected on the basis of a single step equilibrium. Of the five analog-protein complexes studied, only the relaxation processes for the FMN-apoprotein complex are shown not to be well described by Equation 1. It must be added, however, that the relaxation studies on the riboflavin-apoprotein complex are indefinite because of experimental limitations imposed by the small value of $K_d$. The origin of the small discrepancy for the FMN studies is unknown, but is not necessarily related to the negative charge on the flavin since the observed relaxation behavior with lumiflavin 3-ace- tate agrees well with that expected for single step binding. The conclusion that binding of widely different analogs is adequately described by a single step process justifies calculating the value of $k_1$ from $k_2/K_d$ in other cases. Calculation of the activation energies for the dissociation process from the results of the equilibrium and association studies is also validated. This allows the completion of the thermodynamic description for the binding of these flavin analogs by aporiboflavin-binding protein. Small differences in the value of $k_i$ for analog binding coupled with large differences in the $K_d$ values have a consequence in the relationship between $K_d$ and $k_i$ for a single step equilibrium.

$$\log(K_d^{-1}) = \log k_i - \log k_i$$

When $k_i$ is invariant, a plot of $\log(K_d^{-1})$ versus $\log k_i$ is linear with slope of $-1$. The values for $\log K_d$ and $\log k_i$ cover almost 4 decades (Fig. 10C) and the data fall close to a line with the expected slope. Thus, to a considerable degree, the strength of the interaction ($K_d$) depends on the rate of dissociation ($k_i$) of the complex. This result is consistent with a similar observation made by Theorell and Nygaard (6) for the binding of riboflavin, FMN, and FAD to apo-Old Yellow Enzyme.

The importance of using pure analogs in studies such as these cannot be over emphasized. A technique for affinity chromatography of flavin samples on Sephadex G-50 is found to achieve a separation of the flavins from some impurities, but is unable to cleanly separate different flavin analogs. In addition, some of the analogs (e.g., 3,4-dihydroriboflavin and 3-methyl, 8a-morpholinolumiflavin) were found to contain impurities when subsequently analyzed by thin layer chromatography. Accordingly, the binding studies for these analogs may warrant re-examination using samples further purified by preparative thin layer chromatography or an equivalent method.

REFERENCES


Additional references will be found on p. 5616.
Flavin Binding to Riboflavin-binding Protein

Spectral and Fluorescence Properties

Flavin-binding proteins (FBPs) are a class of proteins that bind and transport flavin molecules, which are essential for cellular metabolism. The interaction between flavins and their binding proteins is crucial for understanding various biological processes, particularly in energy metabolism and cell signaling. In this section, we explore the spectral and fluorescence properties of flavin-binding proteins, focusing on the binding of riboflavin to its specific binding protein.

### Materials and Methods

Flavins and FBP were obtained from Sigma Chemical Co., and from Calbiochem. Horse serum-free riboflavin binding protein (HFBP) was obtained from the American Type Culture Collection (ATCC). Samples of flavin mononucleotides (FMN) and flavin adenine dinucleotides (FAD) were prepared by dissolving the respective salts in distilled water. The solutions were then filtered through a 0.22-μm filter to remove any particulates.

### Spectral Analysis

Spectrophotometric studies were performed to determine the absorption and emission spectra of flavin binds to FBP. The absorption spectrum of the flavin-FBP complex was recorded using a UV-Vis spectrophotometer. The emission spectrum was recorded using a fluorescence spectrophotometer.

### Fluorescence Studies

Flavins exhibit fluorescence upon excitation with appropriate wavelengths. The fluorescence properties of flavin-FBP complexes were studied to understand the conformational changes and energy transfer phenomena. The fluorescence spectra were recorded using a fluorescence spectrophotometer, and the quenching experiments were performed to determine the effect of various reagents on the fluorescence intensity.

### Conclusion

The spectral and fluorescence properties of flavin binding to FBP provide insights into the molecular interactions involved in the flavin-FBP complex. These studies are crucial for understanding the biological roles of flavin-binding proteins and their potential applications in various fields, including medicine and biotechnology.
Flavin Binding to Riboflavin-Binding Protein

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B

Figure 1. Homogeneity studies of riboflavin-binding protein. Blue gel electrophoresis at pH 9.5; samples of 2.35, and 100 mg/ml were stained with
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Figure 2. Determination of the concentration of apo-riboflavin-binding protein. 1.0 ml of 1.25 mM riboflavin was titrated with bovine plasma albumin at a solution of 155 μl. The end-point of 155 μl corresponded to a concentration of apoprotein at 4.5 μM.

Figure 3. Job Analysis for the binding of riboflavin. Molar solutions of riboflavin and the apoprotein (both at 1.25 mM) were added in several proportions as indicated. The resultant titrations were plotted as a function of the mole fraction of apoprotein. It is evident that although the apoprotein had been preincubated (5 min at 10°C) with riboflavin, the protein concentration was determined nephelometrically (Miller and M. Hunter, unpublished data).
The binding of flavin derivatives to the riboflavin-binding protein of egg white. A kinetic and thermodynamic study.
J Becvar and G Palmer


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