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 20 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. Association rate constants approaching 3 × 10⁶ M⁻¹s⁻¹ are observed for binding of flavin analogs to aporiboflavin-binding protein at 25 °C and pH 7.0. 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 (1).

To date, the most widely used approach for characterizing the nature of 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 B₃ 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

* Portions of this paper (including "Materials and Methods," the preparation of flavin-saturated riboflavin-binding protein and its conversion to the apoprotein, a presentation of the

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Flavin Binding to Riboflavin-binding Protein

Equilibrium energies for the binding of some flavin analogs by aporiboflavin-binding protein

Fluorescence quenching titrations were conducted in 0.01 M potassium phosphate, pH 7.0.

<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>$1.3 \times 10^{-6}$</td>
<td>-14.7 ± 0.2</td>
<td>-20.3 ± 0.2</td>
<td>-19 ± 1</td>
</tr>
<tr>
<td>Lumichrome</td>
<td>$4.7 \times 10^{-9}$</td>
<td>-12.4 ± 0.2</td>
<td>-17.3 ± 0.1</td>
<td>-17 ± 1</td>
</tr>
<tr>
<td>10-Hydroxyethyl flavin</td>
<td>$9.3 \times 10^{-6}$</td>
<td>-12.0 ± 0.2</td>
<td>-17.3 ± 0.2</td>
<td>-18 ± 1</td>
</tr>
<tr>
<td>Tetraacetylriboflavin</td>
<td>$2.9 \times 10^{-8}$</td>
<td>-12.7 ± 0.1</td>
<td>-18.7 ± 0.3</td>
<td>-20 ± 1</td>
</tr>
</tbody>
</table>

$\Delta G$ (25 °C) was calculated from the first addition of apoprotein (Figs. 1 to 3 and Table I).

RESULTS

Equilibrium Studies on the Binding of Flavins by Aporiboflavin-binding Protein—The results of a fluorescence titration 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 $[F]_b$, $[P]_b$, 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 signifying inhomogeneity in the analog (e.g., the 3,4-dihydriodroflavin 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 NH4NO3 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...
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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 (×) or from CD measurements (○). 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-C1 (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. ×, 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-C1, pH 7.0 (X), 0.1 M sodium acetate, pH 4.1 (○), or 0.1 M sodium acetate, pH 3.8 (□). 

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; \( \Delta G_0 \) (25 °C) was calculated from the interpolated value of \( K_d \) at 25 °C. Each error limit in \( \Delta G_0 \) (25 °C) represents the relative standard deviation of the differences between the observed \( K_d \) at all temperatures and the “best fit” line. \( \Delta S \), (25 °C) was then evaluated from \( \Delta H \) and \( \Delta G_0 \), and the error limit in \( \Delta S \) was set equal to the larger of the error limits in \( \Delta H \) and \( \Delta G_0 \), (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
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

\[ k_t = \frac{2.303 \times [P]_{eq}}{[F]_{eq}^2} = \log \left( \frac{[P]_{eq}}{(F)_{eq}^2 - [P]_{eq}} \right) \]  

where \([PF]\) is the concentration of the complex at time \(t\) and \([PF]_{eq}\) 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 Apoprotein**—The binding kinetics for the reaction of apoprotein with 16 flavin analogs was studied over the temperature range 10–40 °C. Fig. 7 summarizes the results in the form of Arrhenius plots for four representative flavins. In general, curvature was not evident with the exception that the rates of some analogs
Flavin Binding to Riboflavin-binding Protein

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 $\Delta G^\ddagger$ (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 $\Delta G^\ddagger$ (25 °C), and the larger of the error limits on $\Delta H^\ddagger$ or $\Delta G^\ddagger$ was used to set the error limit on $\Delta S^\ddagger$. 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:

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

1:1 dilution does not violate the conditions $[oF] \ll [F]$, necessary to obtain the above equation. Reliable values of $k_{-1}$ are obtained when $k_{-1} \gg k_1([P] + [F])$. 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 $([P] + [F])$, such that $k_{-1} \ll k_1([P] + [F]) < 200 \text{ s}^{-1}$, 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) $K_d > 10^{-5} \text{ M}$, 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 $([P] + [F])$ 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](image-url). 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.
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>Δ$G$ $\ddagger$ (25 °C)</th>
<th>Δ$H$ $\ddagger$</th>
<th>Δ$S$ $\ddagger$ (25 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin $^a$</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>Lumichrome</td>
<td>23</td>
<td>15.6</td>
<td>~25</td>
<td>~33</td>
</tr>
<tr>
<td>10-Hydroxyethyl flavin</td>
<td>2.8</td>
<td>16.8</td>
<td>26.1</td>
<td>31</td>
</tr>
<tr>
<td>Tetracyctethylflavin</td>
<td>92</td>
<td>14.6</td>
<td>24.7</td>
<td>34</td>
</tr>
<tr>
<td>FMN $^a$ $^b$</td>
<td>11</td>
<td>15.8</td>
<td>25.1</td>
<td>31</td>
</tr>
<tr>
<td>3-Methyl lumiflavin</td>
<td>8.8</td>
<td>16.1</td>
<td>20.0</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-Dihydroriboflavin</td>
<td>0.9</td>
<td>17.4</td>
<td>23.9</td>
<td>22</td>
</tr>
<tr>
<td>Deaza riboflavin</td>
<td>0.52</td>
<td>17.9</td>
<td>24.8</td>
<td>30</td>
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<tr>
<td>Isolumiflavin</td>
<td>13.6</td>
<td>15.9</td>
<td>23.1</td>
<td>13</td>
</tr>
<tr>
<td>7,10-Dimethyisooxazine</td>
<td>55</td>
<td>15.0</td>
<td>20.6</td>
<td>18</td>
</tr>
<tr>
<td>10-Methyisooxazine</td>
<td>44</td>
<td>15.2</td>
<td>21.0</td>
<td>20</td>
</tr>
<tr>
<td>7-Chlorolumiflavin</td>
<td>3.4</td>
<td>16.7</td>
<td>24.8</td>
<td>28</td>
</tr>
<tr>
<td>8-Chlorolumiflavin</td>
<td>11.8</td>
<td>16.0</td>
<td>22.8</td>
<td>23</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-morpholium-nolumiflavin</td>
<td>250</td>
<td>14.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3-Dimethylalloxazine</td>
<td>6</td>
<td>16.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The binding of these analogs may not be adequately described by a one-step reversible reaction.

$^b$ These values are subject to additional uncertainty because of the two temperature Arhenius analyses.

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

However, it seems clear that the binding of those analogs which differ only slightly from lumiflavin can be adequately described by a one-step reversible reaction. On this basis, the equilibrium energies in Table II and the activation energies in Table IV can be used to obtain the activation energies for the dissociation of these analogs from the riboflavin-binding protein. These energies are given in Table V.

**DISCUSSION**

The preparation of riboflavin-binding protein used in these studies is greater than 95% homogeneous by disc gel electrophoresis at pH 9 and by SDS gel electrophoresis at pH 7.0. A slight heterogeneity is found by disc gel electrophoresis at pH 6 and by gel isoelectric focusing. Both fractions seen in the electrofocused sample contain bound flavin. The preparation, therefore, appears to be homogeneous with respect to size, but contains a slight charge heterogeneity.

The protein has a PI value of 4.5, a value slightly higher than found previously (8), and a molecular weight of about 30,000 which is in close agreement with published values (8-10).

No evidence is found to support the conclusion of Clagett and co-workers (10-12) that this protein is composed of subunits of 24,000 and 8,000 daltons connected by disulfide bonds. Protein treated with 2-mercaptoethanol migrates during SDS gel electrophoresis as a homogeneous protein of 30,000 daltons. The absence of stainable material migrating with higher mobility indicates the absence of polypeptides with molecular weight less than 30,000.

The apoprotein is easily prepared and is homogeneous with respect to binding, as tested by two criteria. 1) The Scatchard analysis for riboflavin binding shows that the interaction is characterized by an unique association constant over wide ranges in the fraction of both flavin and of apoprotein complexed. 2) Apoprotein fractions obtained by chromatography on sulfoethyl-Sephadex (a method which is likely to separate the differently charged protein molecules seen on electrophoresis at pH 6) show identical behavior in regard to the binding of lumiflavin.

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$^{-10}$ M to greater than 10$^{-7}$ 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 (2) and to Pectostreptococcus elsdenii apoflavodoxin (5) with greater affinity, while flavin adenine dinucleotide binds to apo-D-amino acid oxidase with a $K_d$ value about 2 × 10$^{-7}$ M (3) and FMN to luciferase with the suprisingly 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 Δ$G$, Δ$H$, or TΔ$S$ 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 contrasted by 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 (3), while Azoto bacter vinelandii apoflavodoxin (Sheathina 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 between 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 C4 carbonyl, N5, and the C8 methyl, all stabilize the binding interaction by about 1–2 kcal. The presence of an additional methyl group of C4 has no effect, while methylation of either N or N5 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 (Δ$G$ = −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...
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 in 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-methylisoalloxazine 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 ΔGo 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 plot for the association of flavin analogs to the apoprotein; B, activation entropy-activation enthalpy plot 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), tetraacetylriboflavin (2), FMN (3), deaza riboflavin (4), 10 hydroxylflavin (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-dihydrolumiflavin (13), 7,8-dichlorolumiflavin (14), isolumiflavin (15), 1,3,7,8-tetramethyl 4-methoxyalloxazine (16), 10 methylisoalloxazine (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 $3 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$. These values are quite large and approach the largest 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 morpholino function at position 8. These observations correlate 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 III). 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 $\Delta G^\pm_2$, $\Delta H^\pm_2$, and $T\Delta S^\pm_2$ of 7, 12, and $-5 \text{ kcal/mol}$, 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 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 by aporiboflavin-binding protein demonstrate that the binding process for many analogs can be adequately described by a single step process (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 $K_d$ 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 $k_1$ for analog binding coupled with large differences in the $K_d$ values have a consequence in the relationship between $K_d$ and $k_1$, for a single step equilibrium.

$$\log(K_d)^{-1} = \log k_1 - \log k_2$$

When $k_1$ is invariant, a plot of $\log (K_d)^{-1}$ versus $\log k_2$ is linear with slope of $-1$. The values for $\log k_1$ and $\log k_2$ 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 ($k_1$) 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-morpholino-10methylflavin) 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.
Flavin Binding to Riboflavin-binding Protein

Supplemental Material

The Binding of Flavin Derivatives to Riboflavin-binding Protein of Egg-white, a Nutritional and Therapeutic Study.

JAMES BURGER and L. WARREN FISHER

This supplemental material contains a description of the materials and methods, the preparation of solutions of labeled riboflavin-binding protein, and the comparison to the standard, presentation of the isolated standard and a discussion of the preparation of the standard and the final standard. The presentation is organized into the sections of this text:

Materials and Methods

Riboflavin and PMN were obtained from Sigma Chemical Co., and the preparation of PMN was described riboflavin-binding protein. The methods are summarized in the following sections:

Flavin binding to riboflavin-binding protein from egg-white was performed by incubation of labeled flavin with riboflavin-binding protein at pH 7.0 for 1 hour at 37°C. The mixture was centrifuged and the supernatant was assayed for flavin content.

Equilibrium binding constant

The binding constant for flavin to riboflavin-binding protein was calculated from the following equation:

$$K = \frac{[F]}{[P]}$$

where [F] is the concentration of flavin and [P] is the concentration of riboflavin-binding protein.

The equilibrium binding constant for flavin to riboflavin-binding protein was determined by equilibrium dialysis. The samples were dialyzed against a solution containing riboflavin-binding protein at a constant concentration of 10 µM and flavin at concentrations ranging from 10 nM to 100 µM. The mixture was incubated at 37°C for 1 hour.

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Flavin Binding to Riboflavin-binding Protein

The molecular weight of the protein was determined to be 39,000 by gel electrophoresis (gE). This value is in close agreement with that reported from the stoichiometry of flavin bound (see each paper). Further sedimentation studies and analytical data led to an estimate of 38,500 for the molecular weight of the protein.

Electrostatic focusing of Riboflavin Binding Proteins for 5 hours reveals a slight heterogeneity consistent with the slight charge heterogeneity found in gel electrophoresis. The heterogeneity could also be observed visually at early times of gE electrophoresis via the yellow color of the flavin bands. These results suggest that the Riboflavin Binding Protein used in this study contains a single multiple molecule which is homogenous in size but slightly heterogeneous in total charge.

The isoelectric point of the protein was measured using the gel electrofocusing technique. A series of gels containing proteins were electrofocused simultaneously. The gels were then run on a slab gel apparatus and at a constant current of 30 mA. The isoelectric point of the Riboflavin Binding Protein was found by the study of the position of the protein bands which is homogenous and in slightly heterogeneous in total charge.

The isoelectric point of the protein was determined using the method of Dole et al. (14) using glycine as reference. 9.8 ml of equivalent glycine solution was added to 10 mg of protein. No reference was used. This method does not suit the article's requirements.

The activity of the protein was determined by the method of Dole et al. (15) using a modification of the method of Nurse (16). Using either pancreatic ribonuclease or trypsin these proteins were immobilized on the protein. The activity of a 5 ml of 0.05 M substrate noted. A comparison of the pH value of the equilibrium reaction was noted. The protein in the final stage indicates the protein has a pH value of 4.0, a value slightly higher than that originally determined by Dole et al. (14).

The activity of the Riboflavin Binding Protein was measured by the method of Dole et al. (16) using glycine as reference. 9.8 ml of equivalent glycine solution was added to 10 mg of protein. No reference was used. This method does not suit the article's requirements.

The activity of the protein was determined using the method of Dole et al. (17) using glycine as reference. 9.8 ml of equivalent glycine solution was added to 10 mg of protein. No reference was used. This method does not suit the article's requirements.

No lipid was detected in this protein by the Sudan Black B staining method of Sargent (18).

Determination of Binding Site Concentration

The concentration of binding sites in the apoprotein was determined by titrating freshly prepared riboflavin solutions with apoprotein and monitoring the quenching of the flavin fluorescence (Fig. 2). The total concentration of riboflavin was chosen to be much larger than the value of Kd so that each addition of apoprotein would presumably produce a new binding site as well as a new quenching of the fluorescence of the flavin. The quenching of fluorescence was expressed as a function of the concentration of apoprotein added. The value of the total concentration of apoprotein at the point of half-maximal quenching was calculated using the equation in this study. The value of the total concentration of apoprotein at the point of half-maximal quenching was calculated using the equation in this study.

In a typical experiment, the protein a value of 6.8 and the flavin a value of 26.8 were determined. The total concentration of riboflavin was 26.8 and the total concentration of apoprotein of 6.8. The concentration of the flavin was 26.8. The concentration of apoprotein was 6.8. The concentration of riboflavin was 26.8. The concentration of apoprotein was 6.8.

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Flavin Binding to Riboflavin-binding Protein

Figure 1: Measurement of the concentration of protein-bound flavin by the enhancement of fluorescence. The ordinate represents the fluorescence signal in millivolts, and the abscissa represents the mole fraction of apoprotein added. The protein concentration is constant at 0.25 mg/ml.

Figure 2: Measurement of the molar absorptivity of flavin at 450 nm in the absence and presence of protein. The ordinate represents the molar absorptivity in liters per mole per centimeter, and the abscissa represents the mole fraction of riboflavin.

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The binding of flavin derivatives to the riboflavin-binding protein of egg white. A kinetic and thermodynamic study.


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