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Frontal Gel Chromatographic Analysis of the Interaction of a Protein with Self-Associating Ligands: Aberrant Saturation in the Binding of Flavins to Bovine Serum Albumin[1]

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Frontal gel chromatography is an accurate method to obtain the total free ligand concentration of a protein-ligand mixture in which ligands self-associate. The average number of bound ligands per protein molecule is obtained as a function of the total free ligand concentration. The method was applied to the interaction of bovine serum albumin with self-associating flavins. The binding curves for FMN and FAD leveled off at about 0.7 and 0.5, respectively. These data were simulated well by a binding model where flavins undergo isodesmic indefinite self-association and the monomer alone binds to a single binding site of albumin. The isodesmic association constants of FMN and FAD were (1.7 ± 0.1) × 10^2 and (2.2 ± 0.3) × 10^2 M^{-1}, respectively. The binding constants of the monomer of FMN and FAD were (7.6 ± 0.2) × 10^2 and (3.5 ± 0.2) × 10^2 M^{-1}, respectively. FMN competitively inhibited the binding of FAD to albumin. The affinity to flavins was in the following order at pH 5.8: lumiflavin, FMN, riboflavin, and FAD. The SH modification and the binding of palmitate did not affect the FMN binding to bovine albumin at pH 5.8. As pH increased from 5.8 to 9.0, the affinity to FMN of bovine albumin decreased 3-fold, whereas that of human albumin increased about 80-fold. The present study clearly showed how isodesmic self-association of a ligand can cause apparent saturation of the interaction of a protein with the ligand at levels lower than 1.

Key words: binding system, bovine serum albumin, flavin, frontal gel chromatography, ligand self-association.

Serum albumin is present at about 0.6 mM in the plasma and binds a variety of endogenous and exogenous ligands to play essential roles in their transport, distribution, and metabolism (*1–3*). Albumin is a three-domain protein and can take several conformations with different binding properties depending on pH (*4–6*). A conformational transition at slightly alkaline pH (the N-B transition) is physiologically important because the transport function of albumin is controlled through this transition or one akin to it (*7–11*). Recent crystallographic studies have revealed atomic-level details of binding sites specific for various ligands (*12–14*). Production of each stand-alone domain of albumin has afforded important insights into the N-B transition (*15*, *16*). However, there are discrepancies between the crystallographically revealed binding sites and the binding data obtained for solution-state albumin (*17*, *18*). The nature of the N-B transition and the mechanism of linkages between the transition and binding of ligand are still poorly understood.

Flavin is one of the physiological ligands for albumin (*19–21*). Recently we have found that the interaction of flavin with human serum albumin is strongly linked to the N-B transition (manuscript in preparation); for example the apparent binding constants of riboflavin and FMN increase 20- and 80-fold, respectively, as pH increases from 6.0 to 9.0 (see Fig. 1A for the case of FMN binding), whereas those for warfarin and diazepam increase at most only 3-fold. Therefore elucidation of the mechanism of the linkage between flavin binding and the N-B transition is important to understand the nature of the transition, although it is doubtful that albumin participates in flavin metabolism because there are specific carrier proteins for riboflavin in animal sera (*22–25*).

Many endogenous ligands self-associate to a significant degree at physiological concentrations. For example, fatty acids (*26–28*), bilirubin (*29*, *30*), bile acids (*31*, *32*), haemin (*33*, *34*), and porphyrins (*35–37*) undergo self-association. Of the ligands known to self-associate, the self-association of flavin has been most intensively investigated (*38–43*). Two models have been proposed for flavin self-association: the monomer-dimer equilibrium (*40*, *42*), and the isodesmic indefinite self-association of the monomer (*39*, *43*). Binding systems show various apparently peculiar behaviors when the self-association of ligands is of comparable strength to their binding to a protein, and theoretical studies including ours (*44–47*) have investigated in detail such binding systems and simulated possible behaviors that may be found in real binding systems.

In the present study, we have successfully applied our recently developed system of frontal gel chromatography on a semi-micro scale (*18*) to investigate flavin binding to albu-

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min. Frontal gel chromatography is a rapid and theoretically sound method to obtain directly the total free ligand concentration of a protein-ligand mixture in which ligands undergo self-association. Binding curves for FMN and FAD to bovine serum albumin showed apparent saturation at levels significantly lower than 1. Based on the reported models (*39*, *40*, *42*, *43*) for flavin self-association, we analyzed the anomalous binding data by applying our theoretical treatment (*45*) combined with the nonlinear least squares method. We show that self-association of flavin probably causes aberrant saturation of the binding curves.

Biologically important ligands and receptors (acceptors) undergo self-association, and their physiological functions are regulated by controlling the self-association (*48–54*). To further our understanding of these complex binding systems, frontal gel chromatography may be helpful in providing reliable experimental data.

MATERIALS AND METHODS

Materials—Lumiflavin and warfarin were purchased from Sigma. Riboflavin, FMN, and palmitic acid were obtained from Nacalai Tesque, Kyoto; FAD from Seikagaku Kogyo, Tokyo. All other chemicals were of analytical grade.

Preparation of Albumin—Bovine albumin monomer with an intact Cys34 residue (mercaptalbumin) (*2*) was obtained from Fraction V albumin (Sigma) according to the method of Janatova *et al.* (*55*) by using a column of DEAE-Sepharose CL-6B (Pharmacia). The yields were around 8%. The purified albumin solutions were concentrated to 3–6% (2–3 ml) with a Centriflo CF25 membrane filter (Amicon) and dialyzed two times against 500 ml of 0.138 M sodium phosphate (pH 5.8; ionic strength, 0.16). The albumin concentrations were determined with an $E^{1\%}_{1cm}$ value of 6.67 at 279 nm (*55*), and the molecular weight of 66,430 (*56*) was used to calculate molar albumin concentrations.

The purified albumin contained no detectable oligomers as judged by SDS-PAGE (*57*) without a disulfide-reducing reagent. The sulfhydryl content was determined to be 0.8–0.9 mol per mol of albumin by Ellman's method (*58*) as modified by Janatova *et al.* (*55*). The fatty acid content was determined to be less than 0.2 mol per mol of albumin by HPLC analysis of the derivatives of the extracted fatty acids with 9-anthryldiazomethane (Funakoshi, Tokyo) on a Cosmosil $5C_8$ column (Nacalai Tesque; 4.6 × 200 mm) (*59*).

To confirm whether the purified albumin is intact in terms of ligand binding, the binding of L-tryptophan, a well-characterized ligand for a single site on domain III (*1*), was measured in 0.05 M sodium phosphate (pH 7.4) at 5°C by the Hummel-Dreyer method (see below). By nonlinear least-squares analysis in terms of a hyperbolic binding model, the number of binding sites and the binding constant were obtained to be 1.0 ± 0.1 and 7.1 ± 2.1 × 10^4 M^{-1}, respectively.

Human serum albumin monomer was obtained from Fraction V albumin (Sigma) as described previously (*18*) by using a column of DEAE-Sepharose CL-6B (Pharmacia). The concentration of human serum albumin was determined spectrophotometrically using a molar absorption coefficient at 280 nm of 35,200 M^{-1} cm^{-1}.

Preparation of Modified Albumin—Palmitate-bound albumin was prepared according to the method of Parks *et al.* (*60*). The fatty acid content was determined as described above. Iodoacetate treatment of mercaptalbumin was performed by the method of Noel and Hunter (*61*). The *S*-carboxymethyl albumin contained no free SH-groups.

Preparation of Flavins—Riboflavin was recrystallized from 5% acetic acid. FMN was purified by HPLC on a Cosmosil 10C18-P column (Nacalai Tesque; 10 × 250 mm) (*62*). FAD was used without further purification because no significant contaminant was detected by the same HPLC method as used for the purification of FMN. The concentrations of lumiflavin, riboflavin, FMN, and FAD were determined with the respective molar absorption coefficients (M^{-1} cm^{-1}) of 1.09 × 10^4 at 444 nm, 1.25 × 10^4 at 445 nm, 1.23 × 10^4 at 445 nm, and 1.13 × 10^4 at 448 nm.

Gel Chromatographies—Frontal gel chromatography (*18*) was performed at 25°C on a Bio-Gel P-10 column (Bio-Rad; 5 × 55–90 mm) preequilibrated with 0.138 M sodium phosphate (pH 5.8; ionic strength, 0.16). The flavin–albumin mixtures (2.5 ml) containing 0.3–0.8 mM albumin and 0.02–6 mM flavin were prepared in the same buffer, incubated at 25°C for 10 min, then applied to the column. The elution profile of albumin was determined with a BCA protein assay kit (Pierce), and that of flavin was spectrophotometrically determined. We used Bio-Gel P-10 which has the exclusion limit of 20,000 Da to satisfy the following conditions: $V_P = V_{PL_i} = V_0$ and $V_{L_1} = V_{L_i} > V_0$, where V_0 is the void volume, V_P, V_{P_i}, and V_{L_i} are the corresponding elution volumes of a free albumin, albumin with i molecules of ligands and the free ligand i-mer, respectively (i = 2, 3, ...).

The numbers of moles of flavin bound per mole of albumin, r, is calculated according to the following equation by using experimentally determined values of the total free flavin concentration:

$$r = \frac{L_t - L_{ft}}{P_t} \quad (1)$$

where L_t and L_{ft} are the total flavin and the total free flavin concentrations in monomer units, respectively, and P_t is the total albumin concentration. To obtain all data with a comparable precision over the L_t range examined, we used a higher concentration of albumin (0.3–0.8 mM) as the flavin concentration became higher. The L_t/P_t ratio was usually less than 5 and was maximally 8 at two data points in FAD binding experiments.

Initial slopes of binding curves for lumiflavin, riboflavin, FMN, and FAD were obtained by frontal gel chromatography on a TSK-gel $G2000SW_{XL}$ column (Tosoh; 4.6 × 75 mm) as previously described (*18*). For comparison, Hummel-Dreyer gel chromatography was also performed to obtain the initial slopes by high-speed gel filtration on a short TSK-GEL G3000SW column (Tosoh; 7.8 × 75 mm) as previously described (*63*).

pH-Dependence of the Flavin Binding to Serum Albumin—Both bovine and human serum albumin exist in an equilibrium between the N and B conformers around neutral pH (*4–6*). To examine the presence or absence of linkage between flavin binding and the N-B transition of bovine serum albumin, we determined the apparent nk_1 values (see Eq. 4) for lumiflavin, riboflavin and FMN at pH 5.8 and 9.0 by frontal gel chromatography. The buffers used were 0.138 M sodium phosphate (pH 5.8; ionic strength 0.16) and 0.1 M Tris-HCl (pH 9.0; ionic strength was adjusted to 0.16 with NaCl). The frontal gel chromatography was carried out at 25°C with the flavin-albumin mix-

tures containing 300–500 μM albumin and 20–50 μM flavin. The FMN binding curves for human albumin at pH 9.0 and 5.8 were also obtained by frontal gel chromatography in FMN concentration range of 0–1.2 mM.

Analysis of Binding Data—In a binding system in which a ligand self-associates and a protein has identical and independent binding sites for the ligand, the relation between L_{ft} and r is generally given as follows (*45*):

$$L_{ft} = \sum_{i=1}^{\infty} iK_{L_i}(L_1)^i \tag{2}$$

$$r = \frac{n\sum_{i=1}^{x} ik_i K_{L_i}(L_1)^i}{1+\sum_{i=1}^{x} k_i K_{L_i}(L_1)^i} \tag{3}$$

where the x-mer is the largest polymer of the ligand which can bind to the protein, n the number of binding sites on the protein, k_i the intrinsic binding constant of the ligand i-mer, $K_{L_1} \equiv 1$, K_{L_i} the equilibrium constant for the reaction $iL_1 = L_i$, $i = 2, 3, \ldots$, and L_i is the concentration of the ligand i-mer, L_i. From Eqs. 2 and 3, the initial slope of the binding curve (r *versus* L_{ft} plot) is obtained:

$$\lim_{L_{ft}\to 0} \frac{dr}{dL_{ft}} = nk_1 \tag{4}$$

Thus the nk_1 value can be experimentally determined from the initial slope of the binding curve.

Two models for flavin self-association in aqueous solution have been proposed: the monomer-dimer equilibrium (*40, 42*) and the isodesmic indefinite self-association of the monomer (*39, 43*). We analyzed the binding data on the basis of these models.

(1) Monomer–dimer equilibrium of flavin: From Eqs. 2 and 3, the following equations are obtained in this case (*45*):

$$L_{ft} = L_1 + 2K_{L_2}(L_1)^2 \tag{5}$$

$$r = n(k_1+k_1\sqrt{1+8K_{L_2}L_{ft}} + 4k_2K_{L_2}L_{ft})L_{ft}/ [1+\sqrt{1+8K_{L_2}L_{ft}} + (4K_{L_2}+k_1)L_{ft} + k_1L_{ft}\sqrt{1+8K_{L_2}L_{ft}} + 2k_2K_{L_2}(L_{ft})^2] \tag{6}$$

The asymptotic maximal value of r (r_{max}) becomes as follows:

$$r_{max} = \lim_{L_1\to+\infty} r = \begin{cases} n \text{ (only the monomer can bind)} \\ 2n \text{ (the dimer can bind)} \end{cases} \tag{7}$$

In the special case where albumin has a single binding site for flavins ($n = 1$) and only the monomer of a flavin can bind to the site ($k_1 \neq 0$ and $k_2 = 0$), Eq. 6 becomes as follows:

$$r = \frac{2k_1L_{ft}}{1+2k_1L_{ft}+\sqrt{1+8K_{L_2}L_{ft}}} \tag{8}$$

where $L_{ft} = L_1 + 2L_2$ and $r_{max} = 1$.

(2) Isodesmic indefinite self-association of flavin monomer: In this model, flavin monomer as a basic unit associates sequentially with an equal association constant ($K^*_{L_1}$, isodesmic binding constant) for each step; $K^*_{L_1} = L_i/(L_{i-1}\cdot L_1)$, $i = 2, 3, 4, \ldots$:

$$L_1 + L_1 \overset{K^*_{L_1}}{\rightleftharpoons} L_2$$
$$L_2 + L_1 \overset{K^*_{L_1}}{\rightleftharpoons} L_3$$
$$\vdots$$
$$L_{i-1} + L_1 \overset{K^*_{L_1}}{\rightleftharpoons} L_i$$
$$\vdots$$

Scheme 1

By summation of Eq. 2, the following equation is obtained (*45*):

$$L_{ft} = L_1/(1-K^*_{L_1}L_1)^2 \tag{9}$$

Solving Eq. 9 for L_1 and examining the approximate property of the solution at $L_{ft} = +\infty$, we find:

$$\lim_{L_{ft}\to+\infty} L_1 = 1/K^*_{L_1} \tag{10}$$

Thus the concentrations of ligand monomer and oligomers increase only up to the respective finite maximum values as the total ligand concentration increases infinitely (see Fig. 5). Substituting the limiting value of $1/K^*_{L_1}$ for L_1 in Eq. 3 and noting that $K_{L_i} = K^{*i-1}_{L_1}$, the r_{max} value is obtained as follows:

$$r_{max} = \frac{n\sum_{i=1}^{x} ik_i}{K^*_{L_1} + \sum_{i=1}^{x} k_i} \tag{11}$$

The r_{max} value is smaller than nx. In the special case where only the monomer of the ligand interacts with a single site of the protein, namely $n = 1$, $k_1 \neq 0$, and $k_i = 0$ ($i \geq 2$), Eqs. 3 and 11 become as follows, respectively:

$$r = \frac{2k_1L_{ft}}{1+2k_1L_{ft}+2K^*_{L_1}L_{ft}+\sqrt{1+4K^*_{L_1}L_{ft}}} \tag{12}$$

$$r_{max} = \frac{k_1}{K^*_{L_1}+k_1} < 1 \tag{13}$$

Equation 13 clearly shows that the binding site cannot be saturated with ligands as the ligand concentration increases infinitely.

The binding data (r, L_{ft}) were directly fitted to the various models above using a computer program developed for nonlinear least-squares analysis (*64*). The goodness of fit was evaluated by the following criteria (*64*): (i) the standard deviation of fit (root mean square of the variance of fit), RMS = $\sqrt{SS/(q-p)}$, is small and of the same order of magnitude as the experimental error, where SS is the sum of the squares of residuals, q the number of data, and p the number of parameters. (ii) The plot of the residuals against L_{ft} shows the random distribution of the residuals. (iii) The values of parameters are physically reasonable.

RESULTS

Flavin Binding to Albumin at Low Concentrations of Flavins ($L_{ft} < 1.2$ mM)—Substantial levels of self-association have been found in flavin solutions when the concentrations are higher than 1 mM (*38–43*). We first examined flavin binding to albumin at relatively low concentrations of flavin (less than 0.1 mM and 1.2 mM for binding to bovine and human albumin, respectively) to obtain apparent nk_1 values under as small effects of flavin self-association on binding data as possible.

Figure 1A shows FMN binding to human albumin at pH 9.0 and 5.8. The interaction between human albumin and FMN was strong at pH 9.0 and the binding curve (r *versus* L_{ft} plot) was saturated at the level of 0.78. The result sug-

gested that an intact human albumin molecule has a single binding site for FMN, and that the present preparation of the albumin contained about 20% of somehow modified molecules which lack ability to bind FMN. Human albumin showed a weak affinity to FMN at pH 5.8 and the nk_1 value was 2.4×10^2 M^{-1}, about 80-fold smaller than that at pH 9.0 (1.9×10^4 M^{-1}).

Figure 1B shows initial slopes of the FMN and FAD binding curves for bovine albumin at pH 5.8. The nk_1 values obtained were 761 ± 24 M^{-1} for FMN and 349 ± 15 M^{-1} for FAD on the basis of Eq. 4. For comparison we performed Hummel-Dreyer gel chromatography at pH 5.8 to determine the value of nk_1. The nk_1 values for FMN and FAD agreed well with those obtained by frontal gel chromatography. The values of nk_1 did not depend on the albumin concentration in the range of 0.3–0.8 mM.

The apparent nk_1 values determined at pH 5.8 for lumiflavin and riboflavin were $(1.41 \pm 0.34) \times 10^3$ M^{-1} and 546 ± 22 M^{-1}, respectively. Flavin binding to bovine albumin was further examined at pH 9.0. The nk_1 values at pH 9.0 for lumiflavin, riboflavin, and FMN were 1.1×10^3, 59, and 2.1×10^2 M^{-1}, respectively. The nk_1 value for riboflavin decreased greatly from 5.5×10^2 to 59 M^{-1} as pH increased from 5.8 to 9.0. In contrast to the binding of FMN to human albumin, the affinity of bovine albumin for FMN decreased about 3-fold (nk_1 value from 7.4×10^2 to 2.1×10^2 M^{-1}) with an increase in pH from 5.8 to 9.0.

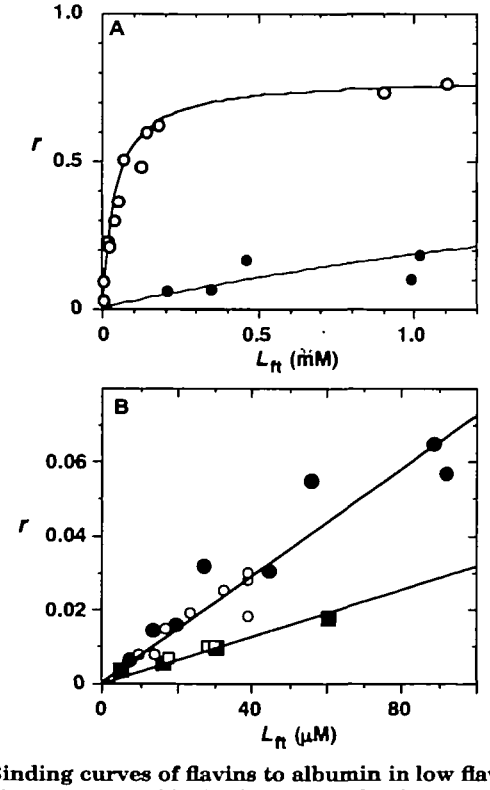

Fig. 1. **Binding curves of flavins to albumin in low flavin concentration ranges.** A: The binding curves for the interaction between FMN and human serum albumin at pH 5.8 (●) and 9.0 (○). All data were obtained by frontal gel chromatography. B: Initial parts of the flavin binding curves were obtained both by frontal gel chromatography (filled symbols) and by Hummel-Dryer chromatography (open symbols). The bovine albumin concentration was 0.3–0.75 mM. ● and ○, FMN; ■ and □, FAD.

The N conformer is predominant at pH 7.5, and the B conformer is the dominant form at pH 9.0 (*4–6*). These results suggested that the binding of flavins is coupled to the N-B transition, and that the N conformer of bovine albumin has higher affinity for flavins than the B conformer. This means that flavin binding shifts the conformational equilibrium of bovine albumin in favor of the N conformer. In the following experiments, therefore, we examined the binding curves for bovine albumin at pH 5.8 to avoid possible effects of the N-B transition on the binding data.

FMN and FAD Binding Curves for Bovine Albumin Obtained by Frontal Gel Chromatography—The low solubility of lumiflavin and riboflavin prevented the determination of binding data in a wide concentration range. The binding curves for FMN and FAD were obtained at pH 5.8 for bovine albumin by frontal gel chromatography over the wide flavin concentration range of 0.2–5 mM.

Figure 2 shows a typical elution pattern of an FMN–albumin mixture. Albumin was eluted in a frontal pattern with a single plateau region, where the P_t value was the same as that in the applied sample. The elution of FMN showed two plateau regions. The constant FMN concentration in the second plateau region corresponded to the L_{ft} value of FMN in the applied sample.

We carried out the frontal gel chromatographic experiments with a pair of the samples, of which one contained both FAD (0.446 mM) and FMN (1.53 mM) and the other contained only FAD (0.474 mM). The total albumin concentration was 0.464 mM. The elution patterns of FMN and FAD were simultaneously obtained by HPLC analyses (*62*) of the individual flavin concentrations in the eluent. The inset in Fig. 2 shows the result: the frontal regions of FAD in the absence and presence of FMN. In the presence of 1.53 mM FMN, the L_{ft} and r values for FAD were 0.428 mM and 0.039, respectively, and those without FMN were 0.397 mM and 0.17. The r value in the absence of FMN was

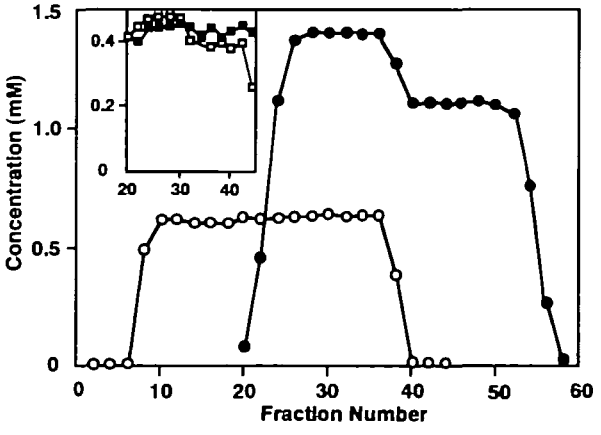

Fig. 2. **Frontal gel chromatography of an FMN-albumin mixture on a Bio-Gel P-10 column (5 × 61 mm) at 25°C.** The sample (2.5 ml) containing 0.626 mM bovine albumin and 1.41 mM FMN was applied to the column preequilibrated with 0.138 M sodium phosphate (pH 5.8). ○, bovine albumin; ●, FMN. The inset shows the elution patterns of an FAD-albumin mixture in the absence and presence of FMN. One applied mixture (□) contained 0.464 mM albumin and 0.474 mM FAD. The other (■) contained 0.464 mM albumin, 0.446 mM FAD, and 1.53 mM FMN.

about 4 times larger than that in the presence of FMN. On the other hand, the L_{ft} and r values for FMN were 1.35 mM and 0.39, respectively: the r value was slightly lower than that of 0.43 in the absence of FAD (Fig. 3A). These results suggested that the two flavins competitively bind to the same site of albumin.

Figure 3A shows the binding curve for FMN. The binding appeared to be saturated at L_{ft} of above 3 mM. The Scatchard plot was almost linear, and the apparent r_{max} was found to be about 0.7 by linear extrapolation to the r axis (Fig. 4A). Figure 3D is the binding curve of FAD. Binding was almost saturated at L_{ft} of above 3 mM, and the apparent r_{max} was estimated to be about 0.5 from the Scatchard plot (Fig. 4C). Both binding curves for FMN and FAD showed apparent saturation at much lower levels than 1, and the saturation levels differed significantly between them. Thus, the present flavin binding system cannot be described by a simple 1:1 hyperbolic mechanism.

Under normal physiological conditions, albumin binds about 2 mol of fatty acid per mol of protein. We prepared palmitate-bound albumin (1.7–2.0 mol palmitate per mol protein) and examined FMN binding to the albumin. When the L_{ft} values were 0.907 and 2.13 mM, the r values were 0.35 and 0.53, respectively (the data points indicated with arrowheads in Fig. 3A). The binding of palmitate did not affect FMN binding to albumin.

The SH-group of Cys34 residue is often chemically modified under physiological conditions. We prepared *S*-carboxymethyl albumin and examined the interaction of FMN with the modified albumin. The r value for FMN was 0.41 at 1.20 mM of L_{ft} (the data point indicated with the arrow in Fig. 3A). The sulfhydryl group modification also did not change the FMN binding activity of albumin.

Analyses of the FMN Binding Curve—We analyzed the FMN binding data (Fig. 3A) by curve-fitting based on models as described in "MATERIALS AND METHODS." The results are summarized in Table I.

1) Hyperbolic binding model: As expected, the 1:1 hyperbolic binding did not fit the data, resulting in the very large RMS value of 0.052 (the broken curves in Figs. 3B and 4B). Assuming that the albumin preparation contained inactive albumin species lacking the flavin-binding activity, the model fitted the data well with n = 0.72. This means that about 30% of the albumin lacked FMN-binding ability, in the same way that the human albumin preparation contained about 20% of albumin molecules lacking FMN-binding ability (Fig. 1A). This model may be excluded for the following reason. The best-fit n value for FAD binding with

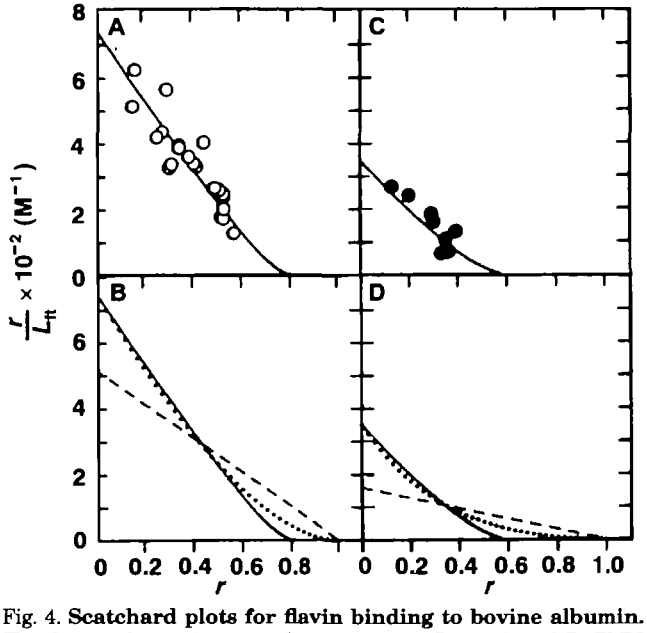

Fig. 4. **Scatchard plots for flavin binding to bovine albumin.** The data and notations are the same as in Fig. 3. A and B, FMN binding; C and D, FAD binding.

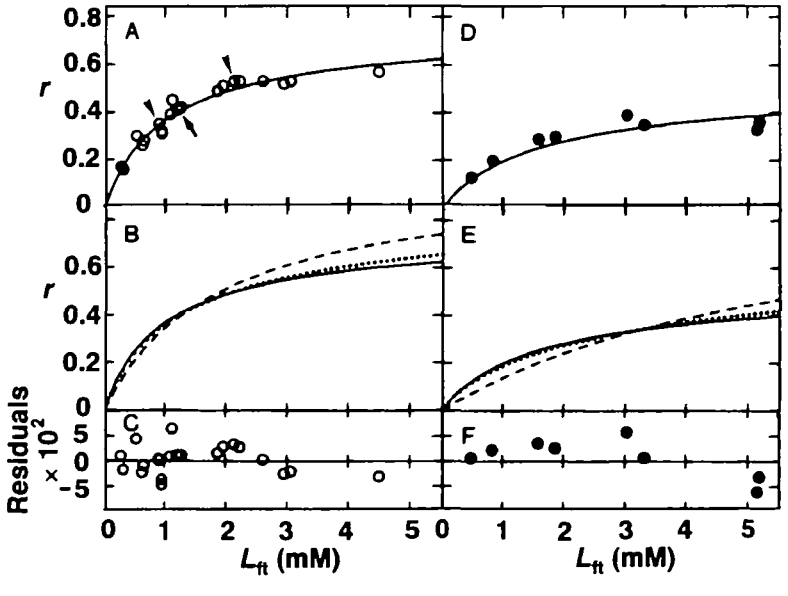

Fig. 3. **Binding curves of FMN and FAD to bovine albumin at 25°C.** The data were obtained by frontal gel chromatography. The albumin concentration was in the range of 0.23–0.82 mM. A: FMN binding curve. The solid curve is the best simulation calculated with Eq. 12 (isodesmic self-association model) with n = 1, x = 1, k_1 = 760 M^{-1}, and $K^*_{L_1}$ = 170 M^{-1} (see text and Table I). The arrowheads indicate the FMN binding data to palmitate-bound albumin (the molar ratio of bound palmitate to albumin was 1.7–2.0). The arrow shows the FMN binding to iodoacetate-treated albumin. B: The best-fit curves for FMN binding based on the three models are compared. The solid curve is the same isodesmic indefinite self-association model as in A; the dotted curve, the monomer-dimer equilibrium model; the broken curve, the hyperbolic binding model. In all models the number of binding sites is 1. All the values of the parameters used for calculation are given in Table I. C: The residuals were plotted against the total free FMN concentration for the isodesmic indefinite self-association model. D: The FAD binding curve. The solid curve is the best simulation calculated with Eq. 12, with n = 1, x = 1, k_1 = 349 M^{-1}, and $K^*_{L_1}$ = 218 M^{-1} (Table II). E: The best-fit curves for FAD binding based on the three models are compared. The notations for the curves are the same as in the case of FMN (Fig. 3B). All the values of the parameters used are given in Table II. F: The residuals were plotted against the total free FAD concentration for the isodesmic indefinite self-association model.

this model was 0.42 (Table II), which differed greatly from the value of 0.72 for FMN binding. Because the two flavins are expected to bind at the same site on albumin molecule, as described above, the n values for FMN and FAD should be equal if this model is valid.

2) Monomer–dimer equilibrium model: As shown in Fig. 1B, the monomer of FMN could bind to albumin. We therefore fitted the data to Eq. 6 under the condition of $k_1 \neq 0$. We first considered a model with $n = 1$ and $k_2 = 0$. As shown in Figs. 3B and 4B (the dotted curves), this model fitted the data well with $k_1 = 9.0 \times 10^2$ M^{-1} and $K_{L_2} = 4.3 \times 10^2$ M^{-1}. The distribution of the residuals in the model was random (data not shown) and the value of k_1 agreed fairly well with that of 7.6×10^2 M^{-1} obtained by the initial slope of the binding curve (Fig. 1B). The model also fitted the data with $K_{L_2} = 2.4 \times 10^2$ M^{-1} using this experimentally determined k_1 value. However, both K_{L_2} values calculated with this model, 4.3×10^2 and 2.4×10^2 M^{-1}, were much larger than the reported values of 118 M^{-1} (*34*) and 140 M^{-1} (*40*).

Next, we attempted to fit the data to the model where both the monomer and dimer can bind, $k_1 \neq 0$ and $k_2 \neq 0$. In all cases examined (the results not shown), the calculated k_2 values were negative and thus physically meaningless. Furthermore, when multiple binding sites for flavin were postulated ($n \geqq 2$) the parameters could not be uniquely determined.

3) Isodesmic indefinite self-association model: We first examined the simplest case according to Eq. 12, where $n = 1$, $k_1 \neq 0$ and $k_i = 0$ for $i \geqq 2$. This model fitted the data very well with $K^*_{L_1} = 1.9 \times 10^2$ M^{-1} and $k_1 = 7.9 \times 10^2$ M^{-1}. The k_1 value agreed well with that of 7.6×10^2 M^{-1} independently obtained by the initial slope of the binding curve (Fig. 1B). The model also fitted the data with $K^*_{L_1} = 1.7 \times 10^2$ M^{-1}, using the experimentally determined k_1 value of 7.6×10^2 M^{-1}. The residuals were randomly distributed and did not appreciably depend on L_{ft} (Fig. 3C). The $K^*_{L_1}$ values, 1.7×10^2 and 1.9×10^2 M^{-1}, were consistent with the reported values of 150–360 M^{-1} (*31*). For comparison, more complex cases were considered as follows: the binding of higher oligomers of FMN to albumin and/or multiple binding sites on albumin ($n \geqq 2$). For example, we examined the models where both the monomer and dimer can bind and $n = 1$ or 2, and the models where $k_i = 0$ for $i \geqq 2$ and $n \geqq 2$. However, the quality of fitting was not improved, and in all cases examined, the obtained $K^*_{L_1}$ values were significantly larger than those for the simplest case, as expected from the relation Eq. A1 in "APPENDIX," or the k_2 values were negative.

Taken together, the above results suggest that the monomer of FMN undergoes isodesmic indefinite self-association ($K^*_{L_1} = 1.7 \times 10^2$ M^{-1}) and only the FMN monomer can bind to a single site on albumin molecule ($k_1 = 7.6 \times 10^2$ M^{-1}).

Analyses of the FAD Binding Curve—We analyzed the binding curve for FAD (Fig. 3D) in the same way as the FMN binding curve, and the results are summarized in Table II.

1) Hyperbolic binding model: Like the FMN binding, the model fitted the data well with $n = 0.42$ and $k_1 = 1.3 \times 10^2$ M^{-1}. This means that about 60% of the albumin lacked FMN-binding ability, and this model may be excluded for the same reasons as described for the FMN-albumin interaction.

2) Monomer–dimer equilibrium model: As is obvious on inspection of Figs. 3D and 4C, the longer-distance extrapolation of the r *versus* L_{ft} plot from $r = 0.4$ to r_{max} ($\geqq 1$) led to considerable computational difficulties and uncertainties in the determined values of the parameters. Among the cases tested, only that of $n = 1$ and $k_1 = 3.5 \times 10^2$ M^{-1}, which is based on the value of nk_1 determined from the initial slope of the binding curve (Fig. 1B), the K_{L_2} value could be obtained to be 4.0×10^2 M^{-1}, much larger than the value of 180 M^{-1} obtained by Kharasch and Novak (*40*). The RMS value (0.053) was also large, and the residuals were not randomly distributed (data not shown).

3) Isodesmic indefinite self-association model: As in the case of FMN, Eq. 12 fitted the data well with $K^*_{L_1} = 2.2 \times 10^2$ M^{-1}, using the k_1 value of 3.5×10^2 M^{-1} determined from the initial slope of the binding curve (Fig. 1B) (RMS = 0.043). The inclusion of k_1 as a free parameter for the fit-

TABLE I. **Summary of the fitting of the FMN-albumin binding data to different self-association schemes for FMN.** The best-fitted parameters are given with SD. The values without SD are the fixed parameters. The notations are given under "MATERIALS AND METHODS."

Model	n	k_1 ($\times 10^{-2}$ M^{-1})	K_{L_2} or $K^*_{L_1}$ ($\times 10^{-2}$ M^{-1})	r_{max}	RMS
Hyperbolic binding	1	5.2 ± 0.3		1	0.052
	0.72 ± 0.03	11 ± 12		0.72	0.028
Monomer-dimer equilibrium	1	9.0 ± 1.4	4.3 ± 2.3	1	0.031
	1	7.6[a]	2.4 ± 0.3	1	0.031
Isodesmic indefinite self-association	1	7.6[a]	1.7 ± 0.1	0.82	0.028
	1	7.9 ± 0.7	1.9 ± 0.5	0.81	0.029

[a]The experimental value of nk_1 obtained from the initial slope of the binding curve (see Fig. 1).

TABLE II. **Summary of the fitting of the FAD-albumin binding data to different self-association schemes for FAD.** The best-fitted parameters are given with SD. The values without SD are the fixed parameters. The notations are given under "MATERIALS AND METHODS."

Model	n	k_1 ($\times 10^{-2}$ M^{-1})	K_{L_2} or $K^*_{L_1}$ ($\times 10^{-2}$ M^{-1})	r_{max}	RMS
Hyperbolic binding	1	1.6 ± 0.3		1	0.088
	0.42 ± 0.03	13 ± 3		0.42	0.031
Monomer-dimer equilibrium	1	3.5[a]	4.0 ± 1.1	1	0.053
Isodesmic indefinite self-association	1	3.5[a]	2.2 ± 0.3	0.62	0.043
	1	6.2 ± 1.6	6.0 ± 2.5	0.51	0.033

[a]The experimental value of nk_1 obtained from the initial slope of the binding curve (see Fig. 1).

ting significantly decreased the RMS value (0.033). However, the fitted k_1 value of 6.2×10^2 M^{-1} was larger than that experimentally obtained. In other cases tested, those of binding of both the monomer and the dimer, and/or multiple binding sites in albumin, the fitted values of $K^*_{L_1}$ were too large and the convergence properties of the calculation were poor (data not shown).

The above results together suggest that the monomer of FAD undergoes isodesmic indefinite self-association ($K^*_{L_1} = 2.2 \times 10^2$ M^{-1}) and only the FAD monomer can bind to a single site on albumin molecule ($k_1 = 3.5 \times 10^2$ M^{-1}).

DISCUSSION

In the present study we applied frontal gel chromatography to investigate the interaction between albumin and flavin, and obtained the binding curves for FMN and FAD to bovine serum albumin at pH 5.8, which showed apparent saturation at the levels of 0.7 and 0.5, respectively. The binding curve for the interaction between FMN and human albumin at pH 9.0 was also obtained by frontal gel chromatography, and the curve showed saturation at the level of 0.78. All three binding curves could be fitted well to a hyperbolic binding model supposing that the albumin preparations were heterogeneous in terms of flavin-binding ability. However, the binding data for bovine albumin were also fitted well to a model where isodesmic indefinite self-association of the flavin monomer exists and only the flavin monomer can bind to a single binding site of albumin. The best-fit values for isodesmic self-association constants for FMN and FAD were comparable to those estimated experimentally for flavin solutions (*38–43*).

The nk_1 value for the binding of flavin to albumin was determined as the initial slope of the binding curve obtained by frontal gel chromatography at low flavin concentrations. The nk_1 values for the binding of riboflavin and FMN to bovine albumin decreased 9- and 3-fold, respectively, with the increase in pH from 5.8 to 9.0. On the other hand, the nk_1 value for the binding of FMN to human albumin increased 80-fold with the same increase in pH. These results show the strongest linkage of ligand binding and the N–B transition hitherto reported for albumin. The molecular mechanism of the N–B transition remains unresolved, but identification of the flavin-binding site will contribute significantly to the understanding of this physiologically important transition.

The nk_1 value for bovine albumin was in the following order at pH 5.8: lumiflavin, FMN, riboflavin, and FAD. This suggests that the isoalloxazine ring moiety of flavin interacts with a binding site of albumin to contribute to the binding, and that the ribityl side-chains are in steric conflict with albumin near the binding site for isoalloxazine ring. The nk_1 value for lumiflavin did not change as pH increased from 5.8 to 9.0, whereas those for riboflavin and FMN significantly decreased. The results suggest that the environment of the ribityl side-chains at the flavin-binding site changes depending on pH.

Figure 5, A and B, shows the weight fraction of each oligomer of FMN and the concentration of FMN monomer as a function of the total FMN concentration, respectively. The $K^*_{L_1}$ value of 170 M^{-1} (Table I) was used to calculate the molar fractions. Almost all of the flavin (>88%) is present as the monomer in the low concentration range ($L_t < 0.1$ mM) at which the nk_1 value was determined as the initial slope of the binding curve (Fig. 1B).

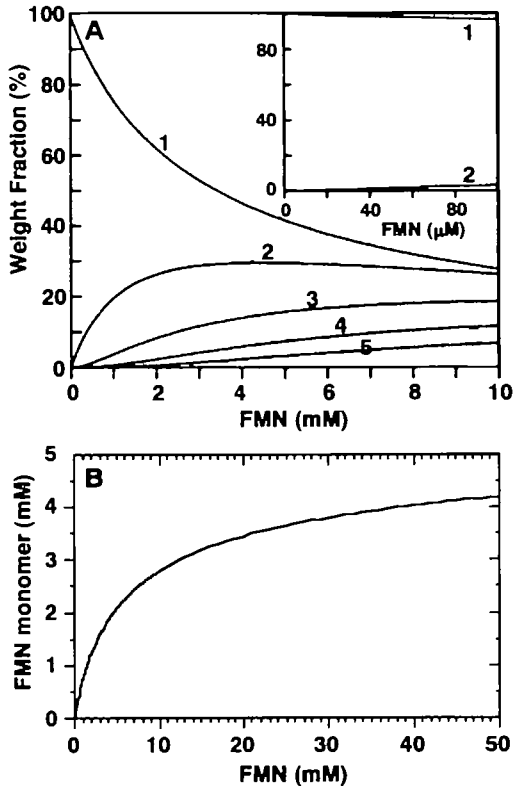

Fig. 5. **Weight fractions of FMN oligomers (A) and the FMN monomer concentration (B) as a function of the total FMN concentration.** The numbers in the figure A indicate the degree of polymerization. The weight fractions were calculated with the isodesmic indefinite self-association model using $K^*_{L_1}$ =170 M^{-1}.

The FMN monomer concentration increases only up to $1/K^*_{L_1}$ with an infinite increase in the total ligand concentration. Because the $1/K^*_{L_1}$ value for FMN is calculated to be 5.9 mM, the binding site of albumin is only 80% saturated at this upper limit of concentration.

We ruled out the possibility that the number of binding sites, n, is greater than 1, because poor convergence properties of the fitting and unrealistic values of the parameters were obtained with higher n values. The following consideration supports the conclusion that $n = 1$. If the correct value of $K^*_{L_1}$ or at least the upper limit of the $K^*_{L_1}$ value, $\overline{K}^*_{L_1}$, is known and the condition of $nk_1 > \overline{K}^*_{L_1} \cdot r_{max}$ is satisfied, the n value should be in the following range (see "APPENDIX") :

$$n \leqq r_{max}/(1 - \overline{K}^*_{L_1} \cdot r_{max}/nk_1) \quad (14)$$

where the values of nk_1 and r_{max} can be experimentally determined. The nk_1 and r_{max} values for FMN were obtained to be 7.6×10^2 M^{-1} and 0.7, respectively. The literature values of the self-association constant for FMN were smaller than 400 M^{-1}. Using these values the right-hand side of the inequality Eq. 14 is calculated to be 1.1. This means that bovine serum albumin has a single binding site for FMN.

When the flavin binding curves were fitted to the monomer-dimer models with $n = 1$ and the nk_1 values obtained from the initial slope of the binding curve, the fitted self-association constant K_{L_2} was always larger than the corresponding constant, $K^*_{L_1}$, obtained from the fitting to the

isodesmic indefinite self-association model with the same values of n and nk_1 (see Tables I and II). This can be explained as follows. If both models fit the binding curve in the whole region, the expected value of r calculated on the basis of Eq. 8 should agree with that based on Eq. 12 within the limits of experimental error for any L_{ft} value. Thus the following equation must be approximately satisfied.

$$\sqrt{1 + 8K^*_{L_2} L_{ft}} = 2K^*_{L_1} L_{ft} + \sqrt{1 + 4K^*_{L_1} L_{ft}} \quad (15)$$

Solving Eq. 16 for K_{L_2}, we obtain

$$K_{L_2} = \frac{1}{2} K^*_{L_1} \left(1 + K^*_{L_1} L_{ft} + \sqrt{1 + 4K^*_{L_1} L_{ft}}\right) \quad (16)$$

Equation 16 clearly shows that K_{L_2} increases monotonically from the minimal value of $K^*_{L_1}$ to infinity as the L_{ft} value increases. In the case of FMN binding, using Eq. 16 and the $K^*_{L_1}$ value of 1.7×10^2 M^{-1}, the K_{L_2} value at $L_{ft} = 5$ mM is calculated to be 3.8×10^2 M^{-1}, about two times larger than that in the low L_{ft} region. This is why the monomer-dimer model led to greater values of the self-association constant than the latter model.

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APPENDIX

Derivation of Equation 14—From Eq. 11, n is expressed as

$$n = r_{max} \left(\frac{\sum_{i=1}^{x} k_i}{\sum_{i=1}^{x} ik_i} + \frac{K^*_{L_1}}{\sum_{i=1}^{x} ik_i} \right) \quad (A1)$$

Both the terms in parentheses in Eq. A1 are monotonically decreasing functions of x. The maximal value of the first term is 1, which is obtained in the case where only the monomer can bind (*i.e.*, $x = 1$), and the maximal value of the second term is $K^*_{L_1}/k_1$. Then, we have

$$n \leqq r_{max} \left(1 + \frac{K^*_{L_1}}{k_1}\right) \quad (A2)$$

The upper limit of $K^*_{L_1}$ is denoted by $\overline{K}^*_{L_1}$, *i.e.*, $K^*_{L_1} \leqq \overline{K}^*_{L_1}$, which may be experimentally estimated. Substituting $\overline{K}^*_{L_1}$ for $K^*_{L_1}$ in Eq. A2 and rearranging the resulting inequality, we find:

$$n \left(1 - \frac{\overline{K}^*_{L_1} \cdot r_{max}}{nk_1}\right) \leqq r_{max} \quad (A3)$$

Under the condition of $nk_1 > \overline{K}^*_{L_1} \cdot r_{max}$, the following inequality is obtained from Eq. A3:

$$n \leqq r_{max} \Big/ \left(1 - \frac{\overline{K}^*_{L_1} \cdot r_{max}}{nk_1}\right) \quad (A4)$$

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