Further Studies on the Riboflavin-Binding Immunoglobulin IgG^{Gar}. Equilibrium and Kinetic Aspects of the Interaction†

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ABSTRACT: The subpopulations of flavin-binding protein IgG^{Gar} which differ with respect to the degree of saturation with riboflavin [Chang, M. Y., Friedman, F. K., Beychok, S., Shyong, J. S., & Osserman, E. F. (1981) *Biochemistry* (preceding paper in this issue)] are characterized in terms of hapten binding activity. Riboflavin fluorescence is used as a sensitive probe of the rate and extent of complex formation. The vacant sites on native IgG^{Gar} reversibly bind up to a total of 2 equiv of riboflavin/mol of protein. Kinetic and equilibrium measurements yield an affinity constant of $(1.7 \pm 0.1) \times 10^9$ M^{-1}. This value is the highest found to date for a hapten–monoclonal antibody system and is a consequence of an unusually low dissociation rate for the complex. The system exhibits maximal binding efficiency between pH 6 and 8. van't Hoff analysis of binding yields $\Delta H = -15$ kcal mol^{-1} and $\Delta S = -9.5$ cal deg^{-1} mol^{-1}. The occupied sites on native IgG^{Gar} irreversibly bind riboflavin under normal reaction conditions. Irreversible denaturation of protein with urea affects the release of riboflavin. The dissociation mechanism is complex and is partially characterized.

In the preceding paper, it was shown that the human monoclonal IgG2(λ) of flavin binding specificity, IgG^{Gar} (Farhangi & Osserman, 1976), is separated into two very similar fractions by ion-exchange chromatography (Chang et al., 1981). One of these, designated fraction A, is nearly saturated with an average of about 2 equiv of riboflavin/mol. The second, slightly more acidic, has little riboflavin bound when isolated, about 0.2 equiv/mol, but binds added riboflavin and other flavin analogues.

It was also noted that an important distinction exists between sites already occupied with riboflavin in the isolated native protein and vacant sites to which riboflavin can be bound, namely, that the latter bind riboflavin reversibly. In contrast, dissociation from the former sites occurs only upon denaturation of the protein.

In this paper, the reversible interaction between riboflavin and IgG^{Gar} is examined, with respect to the equilibrium binding constant and the kinetics of binding. Release of irreversibly bound riboflavin from the native protein was investigated by urea denaturation.

The binding studies reported take advantage of the fact that the fluorescence of riboflavin bound to IgG^{Gar} is completely quenched, provided the protein is not denatured, irrespective of whether the riboflavin is already bound when the IgG^{Gar} is isolated or added to the purified protein (see below).

Materials and Methods

Serum containing IgG^{Gar} was generously supplied by Dr. E. Osserman. The protein was purified and fractionated as described in the preceding paper (Chang et al., 1981). Since the fraction A protein is nearly saturated when isolated, only B protein was used in the equilibrium and kinetic studies.

Equilibrium Measurements. Sample solutions were filtered through 0.4 μM polycarbonate membranes prior to measurements. The fluorescence emission spectra were recorded on a Perkin-Elmer MPF-2A apparatus. Titrations were performed on a Farrand Model MK2 spectrofluorometer.

Protein samples in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.5 (TS buffer), were titrated with riboflavin dissolved in the same buffer. An identical titration procedure was simultaneously carried out on a buffer solution having the same volume as the protein solution. From the increment in fluorescence of the buffer solution after riboflavin addition, the fluorescence intensity per mole of riboflavin can be calculated. Since the fluorescence of riboflavin is 100% quenched when it complexes with protein, the concentrations of free and bound riboflavin in the protein–riboflavin mixture can be deduced. In addition, a riboflavin solution of fixed concentration was used as a standard with which to correct for any drift of the signal caused by instrumental stability. All experiments were carried out under dim light to minimize photodecomposition of riboflavin. The equilibrium constant (K_{eq}) was determined by use of a modified Scatchard plot, in which bound riboflavin is expressed as a concentration in both the abscissa and the ordinate. This amounts to multiplying both sides of the Scatchard equation by the protein concentration (Scatchard, 1949).

pH and Temperature Effects. Titrations were performed at various pH and temperature values. TS buffer was used for all measurements in the temperature range 4–30 °C, and the temperature was controlled by a Model K-2/RD Lauda water bath. For the titrations done at different pH values, protein or riboflavin was first dissolved in TS buffer and then diluted with the appropriate buffer to give the desired concentration and pH. At the end of each titration, the pH of each sample solution was measured.

Stopped-Flow Kinetic Measurements. The concentration of available sites is defined as 1.8 times the protein concentration, since examination of the absorption spectrum of fraction B protein shows that its average riboflavin/protein ratio is 0.2.

Stopped-flow experiments were performed on a modified Durrum-Gibson apparatus (Luchins, 1977) equipped with a temperature-control system. The fluorescence signals were stored and displayed on a Nicolet Explorer 1909A digital oscilloscope and recorded with a Hewlett Packard X-Y recorder (Model 7035B). All measurements were done at 20 °C. Each reaction was repeated several times, and a smooth

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curve was drawn through the superimposed signals.

The association rate constant (k_1) was first determined by carrying out the reaction under conditions such that the concentration of available binding sites was in large excess over the riboflavin concentration, resulting in a pseudo-first-order reaction.

Concentration Jump Experiments. The same apparatus used for stopped-flow measurements was employed in the concentration jump experiments, and the same instrumental settings were used. The protein was first equilibrated with riboflavin at different combining site/riboflavin ratios. Then, the equilibrium state was perturbed by mixing this solution with TS buffer in a 1:1 volume ratio. A new equilibrium was reached through a first-order reaction mechanism. Therefore, the plot of ln ($\Delta F_i/\Delta F$), where ΔF_i and ΔF are the fluorescence changes at time zero and time t, respectively, vs. time produces a straight line whose slope is equal to the relaxation time (τ) of the reaction. Both the association rate constant (k_1) and the dissociation rate constant (k_{-1}) can be calculated by use of the following equation (Eigen & De Maeyer, 1963):

$$1/\tau = k_1([B]_e + [R]_e) + k_{-1}$$

In the equation, $[B]_e$ and $[R]_e$ represent the equilibrium concentrations of the combining site and riboflavin, respectively. The method of Victor et al. (1973) was applied to obtain a best fit for the data without an initial assumption for the value of the equilibrium constant.

Urea Denaturation. A concentrated IgG^{Gar} protein solution was diluted into 2 mL of TS buffer solutions which contained urea at concentrations of up to 6 M. For a standard, the same procedure was applied to a pure riboflavin solution which had the same absorbance at 445 nm as that of the protein. All solutions were protected from light. At different times following initiation of denaturation, the fluorescence emissions of the riboflavin and protein solutions were measured on a Perkin-Elmer Model 2A spectrofluorometer. Part of the fluorescence change occurred too rapidly for direct monitoring by this method, and the progress of the fast phase was therefore examined by the stopped-flow technique. This was carried out on the modified Durrum-Gibson apparatus referred to previously.

Results

Equilibrium Measurements. Riboflavin exhibits a fluorescence peak at 530 nm when excited at 445 nm. The absorption of riboflavin at 445 nm is removed from any protein absorption bands and ensures that the observed fluorescence derives solely from riboflavin. Combination of IgG^{Gar} with riboflavin results in complete quenching of fluorescence and thereby provides a convenient probe to monitor complex formation.

Figure 1 shows a typical binding curve of riboflavin to fraction B. The binding constant of this system was determined by a Scatchard plot, as illustrated in the inset. The plot is a straight line with a single slope, indicating homogeneous combining sites. From the slope, the binding constant (k_{eq}) was calculated to be 1.66×10^9 M^{-1} at 20 °C. From the x intercept, it was found that the fraction has 1.8 available binding sites for riboflavin per molecule.

pH Effect. The pH dependence of the equilibrium constant was measured in the interval pH 4–9. In the range of pH 6–8, the binding constant was not affected by changing the pH and had an average value of 1.7×10^9 M^{-1}. The binding affinity decreased by 50% at pH 5 and 83% at pH 4, falling to a value of 2.8×10^8 M^{-1}; on the alkaline side, the value falls still more sharply, diminishing to 10% of the maximum value at pH 9.

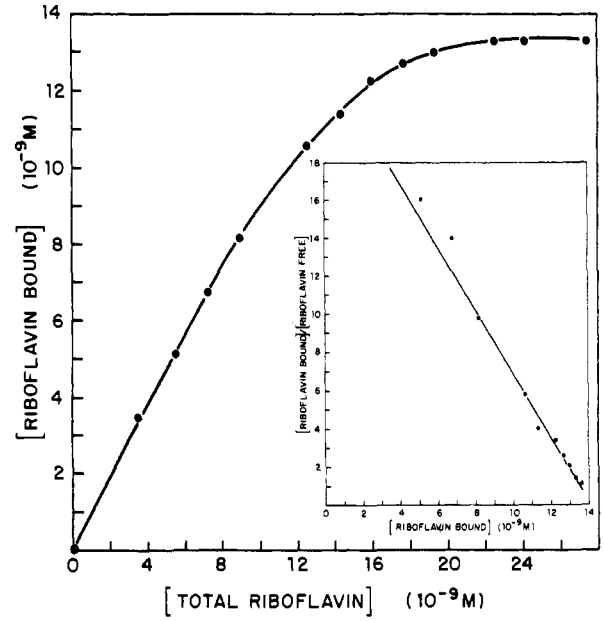

FIGURE 1: Titration curve of B protein with riboflavin. Riboflavin and B protein were both dissolved in TS buffer. Aliquots of riboflavin solution (3.73×10^{-7} M) were added to 2.02 mL of B protein (9.17×10^9 M). Excitation was at 445 nm with an excitation bandwidth of 2.5 nm; emission was recorded at 530 nm with a 10-nm bandwidth. Insert: Scatchard plot of the titration data.

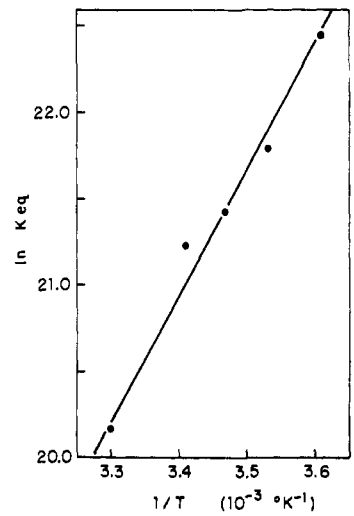

FIGURE 2: Plot of ln K_{eq} vs. $1/T$. Every K_{eq} value is the average of two or three measurements carried out as described in Figure 1.

In terms of stoichiometry, the available combining sites of the IgG^{Gar} protein are relatively constant between pH 4 and 8.5. However, the protein loses about 40% of its binding capacity at pH 9, perhaps owing to partial denaturation of the protein.

Temperature Effect. The equilibrium constant increases as the temperature decreases. K_{eq} at 4 °C is approximately 7.6 times higher than that at 30 °C. Figure 2 displays the temperature dependence in this interval in the form of a van't Hoff plot from which ΔH for this system was found to be −15 kcal mol^{-1}. The entropy, ΔS, was calculated to be −9.5 cal deg^{-1} mol^{-1} by use of the Gibbs function.

Association Rate. The decay of fluorescence after mixing riboflavin with a large excess of protein is shown in Figure 3. The data were analyzed as a simple first-order reaction, and plots of ln ($\Delta F_i/\Delta F$) vs. time were linear. From the variation of the apparent first-order constants with protein concentration, shown in Figure 4, a second-order rate constant of (1.65 ± 0.21) $\times 10^7$ M^{-1} s^{-1} was estimated; this value is close to those

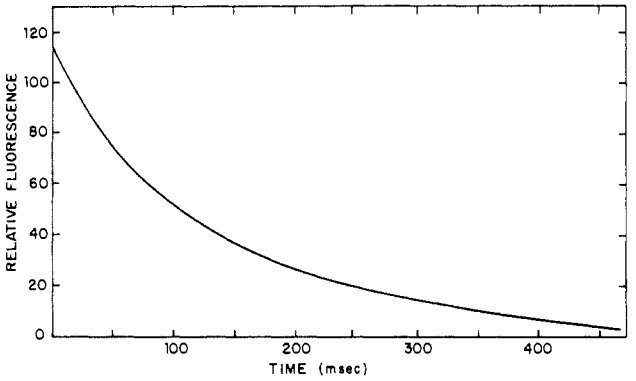

FIGURE 3: Progress of fluorescence decrease upon mixing equal volumes of B protein and riboflavin in a stopped-flow apparatus. The final binding site concentration of B protein was 4.16×10^{-7} M; riboflavin concentration was 4.0×10^{-6} M. TS buffer was used. The full range was 512 ms, and the signal was collected at 5-ms intervals. Temperature was 20 °C. Excitation wavelength = 445 nm, bandwidth = 11.5 nm.

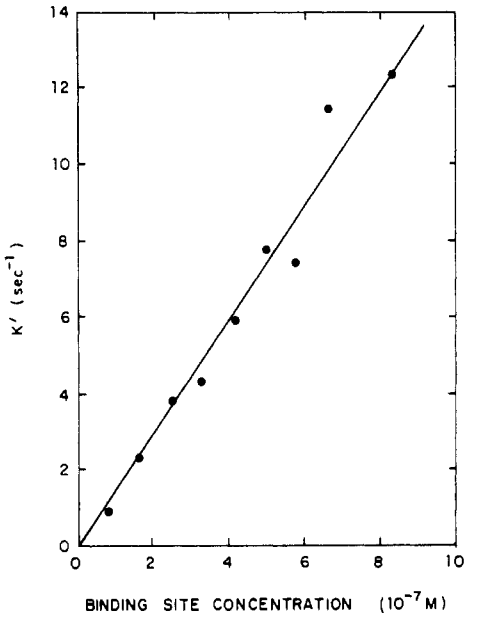

FIGURE 4: Determination of the second-order rate constant under pseudo-first-order conditions. k' vs. binding site concentration is plotted. Every k' value is the average of two measurements carried out by the method described in the text.

found for other antibody–hapten reactions Nisonoff et al., 1975) and is 2 orders of magnitude slower than that of a diffusion-controlled macromolecular association reaction.

Dissociation Rate. A concentration jump relaxation approach was employed to study the dynamic features of the antibody–hapten reaction, in much the same way as it has been used in enzyme–substrate systems (Fisher & Bard, 1969). In this experiment, the equilibrium state of the protein–riboflavin reaction was perturbed by dilution with buffer, and a new equilibrium state was reached. Since the concentration of the protein–riboflavin complex decreases approximately 5% during the process of relaxation, the forward and backward rate constants can be calculated by the relaxation theory. The process results in the increase of fluorescence, owing to release of riboflavin from the protein–riboflavin complex after the dilution.

First-order plots of ln $(\Delta F_i/\Delta F)$ vs. time gave a straight line. The fact that this process is a simple first-order reaction is indicated by the appearance of a single relaxation time. A plot of the inverse relaxation time $(1/\tau)$ vs. the sum of the

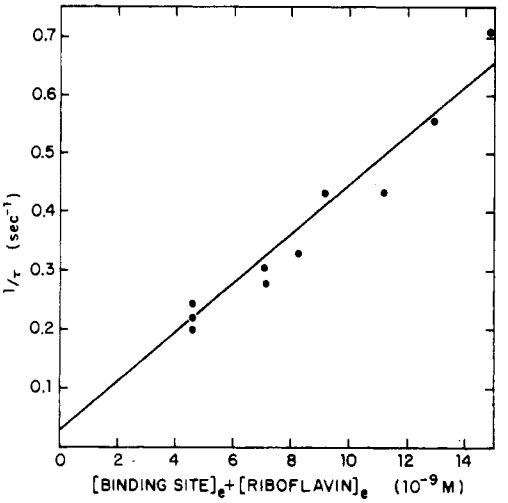

FIGURE 5: Plot of reciprocal τ vs. the sum of equilibrium concentrations of reactants. The value of τ and the method of computation of the straight line are described in the text.

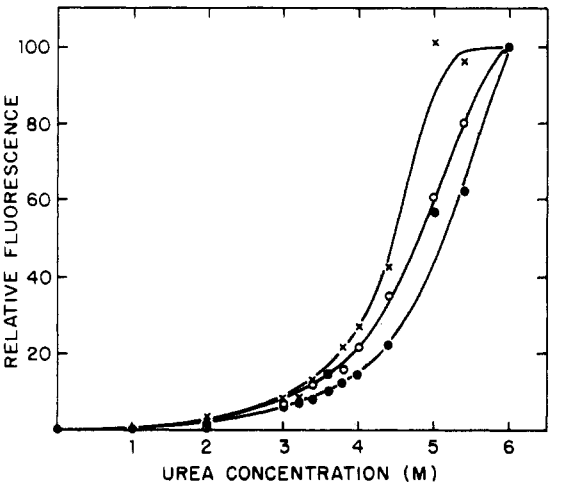

FIGURE 6: Urea denaturation curve for IgG^{Gar}. The fluorescence intensities at 530 nm of urea–protein mixtures (in TS buffer) were measured after 24 (●), 48 (○), and 72 h (×) of denaturation. Excitation wavelength was 445 nm with a bandwidth of 2 nm. The emission bandwidth was 30 nm. All measurements were taken at room temperature.

Table I: Rate Constants of IgG^{Gar} Protein for Riboflavin

method used	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	K_{eq} (M^{-1})
equilibrium measurements			$(1.66 \pm 0.10) \times 10^9$
kinetic measurements	$(1.65 \pm 0.21) \times 10^7$		
concentration jump technique	$(4.2 \pm 0.3) \times 10^7$	$(2.8 \pm 1.8) \times 10^{-2}$	1.50×10^9 [a]

[a] This K_{eq} value is calculated from k_1/k_{-1}.

equilibrium concentrations of the reactants is illustrated in Figure 5. A straight line was obtained with a slope corresponding to the value of the association rate constant (k_1) and the y intercept, which gives the value of the dissociation rate constant (k_{-1}). The ratio of k_1 [$(4.2 \pm 0.3) \times 10^7$ M^{-1} s^{-1}] to k_{-1} [$(2.8 \pm 1.8) \times 10^{-2}$ s^{-1}] yields an equilibrium constant value of 1.5×10^9 M^{-1}, which agrees with the value of 1.66×10^9 M^{-1} measured by the fluorescence titration experiments. The kinetic data are summarized in Table I.

Urea Denaturation. That the interaction between IgG^{Gar} and the riboflavin which is originally bound to it is noncovalent rather than covalent is evidenced by the enhanced fluorescence,

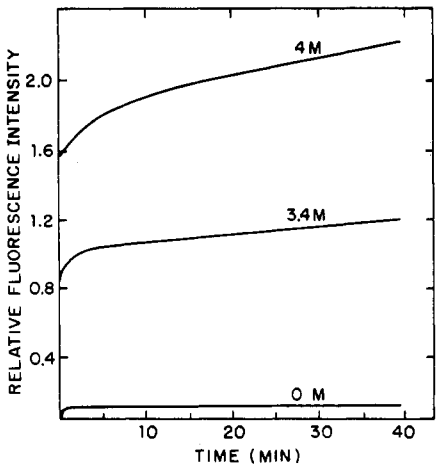

FIGURE 7: Kinetics of urea denaturation of IgG^{Gar}: slow-phase fluorescence enhancement after addition of 4.37×10^{-7} M protein solution to 3.4 and 4 M urea solution (in TS buffer). The instrument settings were the same as those in Figure 6.

indicating release of riboflavin, upon exposure to urea. This riboflavin dissociates from the protein at concentrations greater than 3 M at neutral pH, as seen in the denaturation profile of Figure 6. Protein which had been incubated in 4 M urea for 1 day was subjected to ultracentrifugal analysis and was found to consist of two different species. One species had an $S_{20,w}$ value of 10 S, indicating an aggregated state, while the other species had an $S_{20,w}$ of 6.0 S, which is close to that of native IgG. These results suggest that riboflavin release is accompanied by denaturation of protein and that denaturation is an irreversible process. Consequently, the increase in fluorescence of a protein–urea solution can be used to gauge the extent of protein denaturation.

The sigmoidal denaturation curve illustrated in Figure 6 is typical of a one-stage denaturation process. Nonetheless, it does not rule out the possibility of the existence of intermediates which would be detectable by kinetic analysis (Pace, 1975). The extent of denaturation reaches its maximum value after 3 days and has a midpoint at 4.5 M urea. Denaturation of IgG^{Gar} by urea is thus slow, and the protein is relatively resistant to urea denaturation because complete denaturation occurs only at high urea concentrations.

The rate of denaturation at several different urea and protein concentrations was estimated by following the fluorescence change of the urea solution after addition of protein. Figure 7 depicts a sharp increase of fluorescence in the first few minutes followed by a slower increase over a period of time. The slow-phase data obey first-order kinetics with an apparent rate constant of 1.1×10^{-4} s^{-1} for 3×10^{-7} M protein at 4 M urea. This value should be considered as an order of magnitude only, because the order of reaction with respect to urea and protein was not established. The kinetic irreversibility of denaturation was demonstrated by the following evidence: 1 min after addition of protein to a solution of 6 M urea, the mixture was diluted with TS buffer to give a final urea concentration of less than 2 M, in which the protein has been shown to retain riboflavin; the fluorescence decrease is proportional to the amount of dilution, indicating that the protein is not capable of recombining with riboflavin.

The fast-phase reaction was investigated by the stopped-flow method. An exponentially increasing fluorescence curve was observed and analyzed by the Guggenheim method because the final value was uncertain. At 4 M urea, a rate constant of 0.33 ± 0.05 s^{-1} was obtained; at 5.7 M urea, a slightly greater value, 0.51 ± 0.08 s^{-1}, was observed. In neither case was there a significant dependence on protein concentration.

It should be pointed out that the fast phase might represent dequenching of fluorescence due to conformational flexibility prior to actual release of riboflavin. Certainly, the experiment does not establish dissociation. In addition, in both phases released riboflavin might be rapidly rebound at reversible sites, so that rate constants may not represent elementary unimolecular reactions for either the fast or the slow phase.

Discussion

The interaction between riboflavin and the available combining sites of IgG^{Gar} has been studied by equilibrium and kinetic techniques, by exploiting the quenching of riboflavin fluorescence upon association with IgG^{Gar}. Complete or virtually complete fluorescence quenching has been seen in many flavin–protein reaction systems and is due to the interaction between aromatic amino acid moieties, probably tryptophan, predominantly, and riboflavin in the ground state, to form a nonexcitable complex (Mackenzie et al., 1969).

Two independent methods gave an equilibrium constant of $(1.7 \pm 0.1) \times 10^9$ M^{-1} for riboflavin binding to fraction B, the highest thus far observed for a hapten–monoclonal antibody reaction system. With the consideration that 0.2 mol of riboflavin/mol of protein was found in the B protein (Chang et al., 1981), the available 1.8 combining sites per protein molecule obtained from the titration data confirmed that this protein can be saturated by adding excess riboflavin to reach a maximum riboflavin/protein ratio of 2.0, which is consistent with the stoichiometry of hapten–antibody complexes found in immunoglobulins. Scatchard analysis revealed homogeneous combining sites in both fractions and no evidence of cooperative interaction in riboflavin binding.

The enthalpy found in this reaction is close to the average value obtained in other related enthalpy-driven reactions, such as those of anti-dinitrophenyl antibodies with haptens, and of several mouse myeloma proteins with dinitrophenyl or phosphorylcholine derivatives. On the other hand, a dominant entropic contribution was found to provide the driving force for the interaction of anti-naphthaquinone with hapten derivatives (Johnston et al., 1974), as well as that of a Waldenstrom IgM and its hapten (Ashman & Metzger, 1969; Barisas et al., 1971). In none of these interactions, however, was the free energy of interaction great. In the present case, the strong affinity between protein and riboflavin must be a consequence of the relatively large number of contacts in the binding, reflecting the occupation of the entire site by the hapten, the favorable enthalpy probably arising from hydrogen bonding and electrostatic interactions.

The importance of such bonds is suggested by the pH dependence of the equilibrium constants, which fall off on either side of pH 6 and 8. Below pH 6, the effect is probably due to protonation of carboxyl and/or histidine groups. It should be remembered that while riboflavin is neutral below pH 8.5 the energy of maintaining a histidine residue uncharged below its pK_a, as might be required for riboflavin binding, increases with decreasing pH.

An alternative possibility is that the protein denatures below pH 6. This is unlikely because even prolonged dialysis against the lower pH value of 4 did not dissociate riboflavin from protein.

On the alkaline side of the zone of constant binding affinity, the sharp drop in the binding constant may be due to an ionization either in the protein or in the riboflavin. In certain redox states (Massey et al., 1969), the proton at position N-3 of the isoalloxazine ring is reported to dissociate with a pK_a above 8.5. However, there is no evidence of such an ionization in IgG^{Gar}, and only oxidized riboflavin appears to be present,

with an expected pK_a near pH 10 (Bruice, 1976).

An appealing general hypothesis of riboflavin binding results from the proposal of Massey et al. (1969) of charge separation in the isoalloxazine ring. Under this hypothesis, the combining site on the IgG^{Gar} protein may be composed of two oppositely charged centers. At pHs lower than 6 or higher than 8.5, a change in charge either in the protein or in the riboflavin would introduce an unfavorable interaction in the combining site and result in decreasing binding affinity.

The association rate constant obtained in the system studied is similar to those found in other antibody–hapten systems (Nisonoff et al., 1975). This high association rate constant implies a low energy barrier for activation and is 2 orders of magnitude lower than the value of diffusion-controlled reactions involving macromolecules in biochemical reactions.

Although most antibody–hapten systems have similar association rate constants, their equilibrium constants vary in the range of 10^4–10^8 M^{-1}. The dissociation rate constant is therefore the principal variable governing the binding strength of antibody and hapten. Indeed, our result implies that the high equilibrium constant found in the reaction studied is a consequence of the small dissociation rate constant, as evidenced by a dissociation half-time of 1–4 s.

Finally, as a concluding comment on the preceding paper, as well as the present one, we do not know whether there is a relationship between the irreversibility of riboflavin binding to the native protein and the in vitro irreversibility of reduction when accompanied by chain separation. Several lines of evidence appear to point to the requirement of a complete binding site contributed by both heavy and light chains (failure of L_2 and H_2 dimers and aggregates to bind riboflavin). We are pursuing this problem by investigating the reconstitution and flavin binding of variable region fragments. Elsewhere, it will be shown (J. Sen and S. Beychok, unpublished experiments) that there are similarities in sequences of the combining site of IgG^{Gar} and of other riboflavin binding sites in unrelated proteins. Continued investigations may then help to define the essential features of a "flavin binding site" and shed light on the fascinating question of how such a site became incorporated into a human monoclonal immunoglobulin.

Acknowledgments

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