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Monoclonal antibodies against two electron reduced riboflavin and a quantification of affinity constants for this oxygen-sensitive molecule

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In order to create a protein environment that binds preferentially to the two-electron reduced form of flavin, monoclonal antibodies have been raised against a reduced flavin derivative. Due to the low fluorescence quantum yield and visible light absorption and to the instability of reduced flavin in an aerobic environment, it is not possible to determine the affinities of these antibodies for two-electron-reduced flavin using standard techniques. Because of its sensitivity, time-resolved fluorescence can be used to overcome this problem. This technique has been applied to study the binding of two antibodies, an IgG_1 and an IgM, to reduced riboflavin (1,5-dihydroriboflavin) and oxidized riboflavin (riboflavin). The affinity of the IgG_1 is more than 80 times larger for 1,5-dihydroriboflavin than for riboflavin. From analysis of the dynamical parameters of the system it is apparent that the internal motion of 1,5-dihydroriboflavin bound to IgG_1 is much more restricted than that of riboflavin. In contrast, the affinity of the IgM is only slightly higher for 1,5-dihydroriboflavin than for riboflavin and the flexibility of binding of both flavin redox states in the antigen binding site is almost similar.

Keywords: monoclonal antibodies; time-resolved fluorescence; maximum-entropy analysis; reduced riboflavin; affinity constants.

Flavins mediate a variety of chemical reactions including dehydrogenation, electron transfer, activation of molecular oxygen and photochemical reactions. This versatility sets flavoproteins apart from most other cofactor-dependent enzymes, which, in general, each catalyze a single type of reaction (Ghisla and Massey, 1989). One of the most interesting features of the versatile flavin molecule consists of its redox properties which can be modulated by the (protein) environment. The redox active part of the flavin is the isoalloxazine ring which can exist in oxidized flavoquinone, one-electron-reduced flavosemiquinone and two-electron-reduced flavohydroquinone states. The redox potentials of both electron transfer steps vary largely among different flavoproteins and depend on the chemical nature of the active site in which the isoalloxazine resides. This property makes the flavin suitable as an electron shuttle in very different redox reactions which explains its widespread occurrence in nature (Muller, 1983). The role of the protein environment in modifying the chemical reactions and the redox properties of the flavin is not yet clear. Creating artificial protein environments with a predetermined affinity for oxidized and reduced flavin and comparing the structural and redox properties of these proteins, might contribute to obtaining more insight to this problem. Monoclonal antibodies (mAbs) are very useful tools for this because the flavoquinone differs from the hydroquinone in its conformation and electronic properties. This enables the generation of antibodies specific for either the reduced or the oxidized form. Shokat et al. (1988) have indeed shown that it is possible to create an anti-flavin antibody that can influence the redox properties of flavin.

Here we report a strategy to obtain a protein environment which binds preferentially the 1,5-dihydroflavin and to develop a method to determine the affinity of such an antibody for this flavin. The generation of mAbs is hampered by the instability of the flavohydroquinone under aerobic conditions. This problem was circumvented through the design of a reduced flavin analogue that was not sensitive to reoxidation. Another problem is that, in contrast to its oxidized form, reduced flavin possesses a low fluorescence quantum yield and visible light absorption (Ghisla et al., 1974). For this reason it is difficult to determine dissociation constants for reduced flavin using standard (fluorescence) techniques. Because of its sensitivity, however, time-resolved polarized fluorescence can be used to overcome this problem. Time-resolved fluorescence is one of the very few methods to obtain quantitative information on the binding of both oxidized and reduced flavin to the antibody molecule. The excited state of a fluorophore, such as flavin, has a lifetime in the (sub)nanosecond range. Since this corresponds to the timescale of rotational and internal motion of the antigen in the antigen binding site, time-resolved fluorescence also gives information about the flexibility of the antigen binding as compared to the overall or segmental motion of the antibody.

EXPERIMENTAL PROCEDURES

Synthesis of hapten and conjugates. Riboflavin (from Sigma) dissolved in dry pyridine was refluxed for 4 h with a

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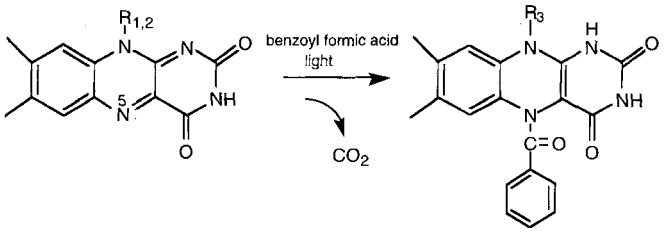
Abbreviations. $\alpha RfmodG_1$, IgG_1 against the modified riboflavin **3a**; αRfmodM, IgM against the modified riboflavin **3a**; MEM, maximum entropy method.



R_1 = $CH_2(CHOH)_3CH_2OCO(CH_2)_2COOH$

R_{2a} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-PTG
R_{2b} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-BSA

R_{3a} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-PTG
R_{3b} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-BSA

Fig. 1. Synthesis of hapten and conjugates. PTG = porcine thyroglobulin.

fivefold excess of succinic anhydride (Bauminger and Wilchek, 1980). After addition of 30% (mass/vol.) toluene, the solvent was removed under reduced pressure. The residue was dissolved in water and purified with DEAE-Sepharose batchwise. The succinylated riboflavin (Fig. 1, R = R_1) was eluted from this material with 1 M NaCl and was conjugated to porcine thyroglobulin (Sigma) or BSA fraction V (Boehringer Mannheim) with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) using general procedures (Fig. 1, R = R_{2a} or R_{2b}, further indicated as **2a** or **2b**, respectively) (Harlow and Lane, 1988). After conjugation, the flavin was modified on the N(5) position by irradiating the flavin-protein conjugate with visible light in the presence of benzoylformic acid under anaerobic conditons yielding the *N*(5)-benzoyl-*N*(10)-ribitylsuccinimide ester flavin (Fig. 1, R = R_{3a} or R_{3b}) (de Kok et al., 1971). The excess benzoylformic acid was removed by dialysis against 0.5% (mass/vol.) ammonium bicarbonate. These hapten-carrier conjugates (indicated as **3a** and **3b** respectively) were freeze dried for storage.

Monoclonal antibody production, selection and purification. Balb/c mice were immunized twice with **3a** (Fig. 1). Spleen cells from these mice were fused to myeloma cells (SP 2/0; Köhler and Milstein, 1975), according to protocols described by Schots and coworkers (Schots et al., 1992). Hybridoma clones producing antibodies against the modified flavin were selected using an enzyme-linked-immunosorbent assay (ELISA) against **3b** (Fig. 1) and BSA alone. For this purpose microtiter plates were coated with 100 µl/well of 10 µg/ml **3b** (Fig. 1) or BSA in phosphate-buffered saline (150 mM NaCl, 50 mM sodium phosphate, pH 7.2). The wells were blocked with 5% skimmed milk powder in phosphate-buffered saline. Mouse antibody was detected by alkaline-phosphatase-conjugated rat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.).

Positive hybridoma clones were cloned until stability, whereafter the cultures were expanded in flasks (150 cm^2). We used Iscove's modification of Dulbecco's medium and low-IgG fetal calf serum for these cultures. mAbs of the IgM isotype were purified by precipitation with 8.5% poly(ethyleneglycol) (M_r 6000, Sigma) followed by gel-filtration chromatography (Superdex 200 prep grade, Pharmacia). IgG_1 antibodies were purified by thiophilic adsorption chromatography (Affi T ™ thiophilic agarose, Biozym). All preparations were judged for purity by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Antibody concentrations were determined by measuring the absorbance at 280 nm assuming $A_{280} = 1.25$ for 1 mg/ml and a molecular mass of 970 kDa for IgM and 150 kDa for IgG_1 (Harlow and Lane, 1988).

Sample preparation for fluorescopic measurements. Since the fluorescence quantum yield of reduced flavin is very low, a minimal concentration of flavin is required to obtain a proper signal. Therefore we chose to titrate of a constant amount of riboflavin or 1,5-dihydroriboflavin with antibody instead of titrating a constant antibody concentration with variable amounts of riboflavin or 1,5-dihydroriboflavin. Different amounts of antibody, up to 0.223 mg/ml for αRfmodG$_1$ and to 0.816 mg/ml for αRfmodM, were mixed with 0.1 µM and 0.05 µM riboflavin, respectively. For measurement of 1,5-dihydroriboflavin, these solutions were deaerated by flushing with argon and riboflavin was reduced with a 30 times molar excess of sodium dithionite. All compounds were dissolved in 50 mM sodium diphosphate pH 8.0 containing 150 mM sodium chloride and filtered through a 0.22-µm filter (Costar).

Time-resolved fluorescence and fluorescence anisotropy measurements. Time-resolved fluorescence decays were measured using the time-correlated single photon counting set up described earlier (Leenders et al., 1993). Vertically polarized light of 444 nm and 450 nm was used to excite 1,5-dihydroriboflavin and riboflavin, respectively. After excitation the polarized fluorescence was monitored using a Schott (Mainz, Germany) interference filter (Schott 557.6 nm, with a full width at half maximum of 12.6 nm) in combination with a Schott cut-off filter (OG515). After each sample, the background of the samples in the absence of antibody and flavin was measured at one fifth of the time of the sample acquisition time. Erythrosin B served as a reference compound to yield the dynamic instrumental response function of the set-up (Vos et al., 1987). Erythrosin B shows a single exponential fluorescence decay time of 80 ps at 20 °C which was the sample temperature used. The data were collected in a multichannel analyser (Nuclear Data model ND66) using 1024 channels/experimental decay with a time spacing of 30 ps/channel. For the registration of the fluorescence decays the excitation energy was chosen to yield a frequency of fluorescence photons of 30 kHz. In this way, the signal/noise ratios in the fluorescence decays of riboflavin and 1,5-dihydroriboflavin were comparable.

Data analysis and calculation of the affinity constant. The fluorescence decays were analyzed in terms of a continuous distribution of decay times by means of the maximum entropy method (MEM; Maximum Entropy Data Consultants Ltd, Cambridge, UK) of analysis yielding the fluorescence lifetime and rotational correlation time distributions of the free and bound riboflavin and 1,5-dihydroriboflavin (Livesey and Brochon, 1987; Brochon, 1994; Leenders et al., 1993). This method recovers the optimal spectrum of decay times by maximizing the Shannon-Jaynes entropy and minimizing the χ^2 statistics and has the advantage that a unique solution is found with no *a priori* knowledge of the decay model.

The time-resolved fluorescence anisotropy [$A(t)$] of a fluorescent molecule in a protein can be adequately described by an expression which assumes two independent rotational motions: a rapid internal motion of the flavin in the antigen binding site (ϕ_{int}) and a slower motion due to the overall or segmental motion of the antibody (ϕ_∞) (the subscript is used to indicate that this time is infinitely long on the experimental timescale) (Szabo, 1984):

$$A(t) = [\beta_1 \exp(-t/\phi_{int}) + \beta_2] \exp(-t/\phi_\infty). \quad (1)$$

We can define a so-called order parameter S for a fluorescence probe attached to the protein. When there is no internal probe motion, the probe rotates together with the whole protein and S is equal to unity. Depending on the freedom of internal probe

motion relative to protein rotation, S will be less than unity. The order parameter S can be obtained from Eqn (2) (see, for example Bastiaens et al., 1992):

$$S = [\beta_2/(\beta_1 + \beta_2)]^{1/2}. \quad (2)$$

From S we can estimate the displacement angle ψ over which the probe can move over the line of attachment to the protein (which is considered to be the local symmetry axis of the potential well in which the probe moves; a further assumption is that the emission transition moment is parallel to this symmetry axis) according to:

$$S = [1/2 \cos \psi (\cos \psi + 1)]^{1/2}. \quad (3)$$

The internal correlation time ϕ_{int} is inversely related to the diffusional rate constant $D_\perp$ via:

$$D_\perp = (1 - S^2)/(6 \phi_{int}). \quad (4)$$

The fraction of bound 1,5-dihydroriboflavin was calculated from anisotropy decays using a fractional analysis approach (Pap et al., 1995). We assumed that the system is in binding equilibrium and consists of a mixture of only two states: bound and free 1,5-dihydroriboflavin. Provided that the decay curves corresponding to free 1,5-dihydroriboflavin and of a sample where the majority of the flavin molecules is bound are known, decays measured at intermediate binding conditions can be described as a combination of both limiting decays. The fraction of bound riboflavin was calculated from the decrease of the free riboflavin contribution in the rotational correlation-time spectra. The dissociation constant (K_d) was then obtained according to the following equations (written out for riboflavin):

$$K_d = \frac{[\text{riboflavin}]_{free}\,[\text{binding sites}]_{free}}{[\text{binding sites}]_{occupied}} \quad (5)$$

$$[\text{binding sites}]_{free} = n\,[\text{Ab}] - [\text{riboflavin}]_{bound} \quad (6)$$

where n corresponds to the number of binding sites per antibody molecule.

Combination of Eqn. (5) and (6) gives:

$$n\text{Ab}] - [\text{riboflavin}]_{bound} = \frac{K_d \cdot [\text{riboflavin}]_{bound}}{[\text{riboflavin}]_{free}}. \quad (7)$$

Plotting $n[\text{Ab}] - [\text{riboflavin}]_{bound}$ as a function of $[\text{riboflavin}]_{bound}/[\text{riboflavin}]_{free}$ gives the K_d. Since riboflavin and 1,5-dihydroriboflavin are small molecules, all binding sites must be available for binding. Therefore it was assumed that $n = 2$ for αRfmodG$_1$ and $n = 10$ for αRfmodM (Edberg et al., 1972).

RESULTS

Synthesis of hapten and conjugates. For mouse immunization we prepared the N(5)-substituted reduced flavin derivative **3a** (Fig. 1), since the two-electron-reduced flavohydroquinone quickly reoxidizes under influence of oxygen. The conjugates **2a** and **2b** (Fig. 1) were obtained by conjugation of the succinylated riboflavin to porcine thyroglobulin and BSA resulting in molar ratios of 44:1 for the former and 6:1 for the latter. After reduction in the presence of benzoyl formic acid, the *N*(5)-benzoyl-*N*(10)-ribitylsuccinimide ester flavins **3a** and **3b** (Fig. 1) both showed an absorption spectrum that is characteristic for reduced flavin.

Monoclonal antibody production, selection and purification. After immunization of mice with conjugate **3a** and generation of mAbs, the resulting hybridomas were first screened for binding to the BSA-conjugate **3b** as well as to BSA alone, and then for binding to the BSA-riboflavin conjugate **2b** (Fig. 1). 16 mAbs bound to **3b** and **2b** and not to BSA alone. No significant difference in binding to the conjugates **2b** and **3b** could be detected, so that at this point we had no indication whether these mAbs indeed bound the reduced flavin analogue preferentially. Two antibodies of different isotype, the IgG$_1$ αRfmodG$_1$ and the IgM αRfmodM, were chosen for further investigation. These hybridoma clones were grown in larger amounts and purified from tissue culture supernatants.

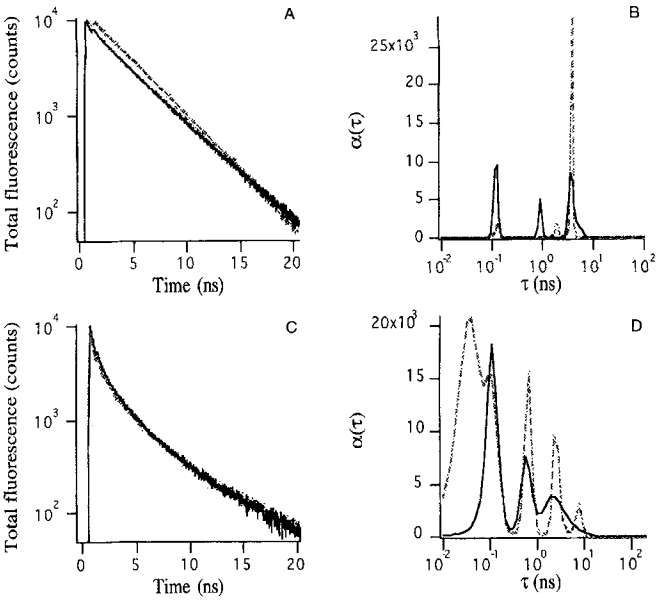


Fig. 2. Experimental total fluorescence decays and fluorescence lifetime distributions of free and αRfmodG$_1$-bound riboflavin and 1,5-dihydroriboflavin. Broken curves reflect free riboflavin (A, B) or free 1,5-dihydroriboflavin (C, D). The solid curves reflect bound species in all cases. In all experiments the residuals of the fit were randomly scattered around zero, indicating an optimal fit.

Time-resolved total fluorescence measurements and analysis. To study the binding of αRfmodG$_1$ to riboflavin we measured 0.1 μM riboflavin incubated with antibody concentrations ranging over 0−1.5 μM. To investigate binding of 1,5-dihydroriboflavin to αRfmodG$_1$ the riboflavin in these samples was reduced. Examples of experimental total fluorescence decays of free and antibody bound riboflavin and 1,5-dihydroriboflavin are shown in Fig. 2A and C. The fluorescence of antibody bound riboflavin exhibits an initial faster decay than the fluorescence of free riboflavin. The decay of free and bound 1,5-dihydroriboflavin shows much more heterogeneity. However, the fluorescence decay characteristics of free and bound 1,5-dihydroriboflavin hardly differ.

The total fluorescence decays are analysed using MEM. The lifetime distribution patterns for both free and antibody bound riboflavin and 1,5-dihydroriboflavin are shown in Fig. 2B and 2D. The corresponding average fluorescence lifetimes obtained are listed in Table 1. Free riboflavin has a major lifetime of 3.7 ns and two small contributions of 1.7 ns and 0.13 ns (Fig. 2B, broken line). The two small contributions are probably due to self-quenching as a result of a tendency of self-association of riboflavin molecules (note that riboflavin does not dissolve in water very well). This is confirmed by the fact that a lower riboflavin concentration gives a longer average fluorescence lifetime (Table 1). Upon addition of increasing amounts of mAb the contribution of the component at 3.7 ns decreases and two contributions around 0.89 ns and 0.11 ns show up

Table 1. Average fluorescence lifetimes of free and antibody-bound riboflavin and 1,5-dihydroriboflavin.

Antibody	Flavin conc.	Average lifetime of	
		riboflavin	1,5-dihydro-riboflavin
	μM	ns	
–	0.05	3.69	2.40
–	0.1	3.29	0.53
αRfmodG$_1$	0.1	2.04[a]	0.88[c]
αRfmodM	0.05	2.04[b]	0.55[c]

[a] Mixture of 32% bound and 68% unbound riboflavin.
[b] Mixture of 24% bound and 76% unbound riboflavin.
[c] Completely bound 1,5-dihydroriboflavin.

(Fig. 2B, solid line) which are responsible for the faster initial decay.

The fluorescence lifetime distribution pattern of 1,5-dihydroriboflavin is more complex than that of riboflavin (Fig. 2D, broken line). This complex pattern can be explained by the presence of multiple, non-planar conformations of reduced flavin, that interconvert into each other (Visser et al., 1991). All these substates cause a more complex lifetime distribution than riboflavin, which has a planar conformation and only shows a main nanosecond lifetime. Free 1,5-dihydroriboflavin shows a broader spectrum of fluorescence lifetimes than antibody-bound 1,5-dihydroriboflavin (Fig. 2D, solid line) which perhaps can be ascribed to a more restricted mobility of bound 1,5-dihydroriboflavin.

Time-resolved fluorescence anisotropy and analysis. From a fluorescence anisotropy decay one obtains information on rotational and internal motions of the fluorophore. After MEM analysis a distribution of rotational correlation times ϕ can be recovered. The experimental fluorescence anisotropy decays and corresponding rotational correlation spectra of free and antibody bound riboflavin and 1,5-dihydroriboflavin are shown in Fig. 3. The rotational diffusion of free riboflavin results in an ultrafast decay of the fluorescence anisotropy (Fig. 3A, broken line). Upon addition of αRfmodG$_1$, the motional freedom of the riboflavin is severely restricted, indicating that binding to this antibody limits the motional freedom of riboflavin (Fig. 3A, solid line). In Fig. 3C the anisotropy decay curves of reduced 1,5-dihydroriboflavin are shown. In the presence of αRfmodG$_1$ the flavin is totally immobilized (solid line). Comparison of the solid curves in Fig. 3A and C suggests that the affinity of αRfmodG$_1$ for 1,5-dihydroriboflavin is significantly higher. Intermediate antibody concentrations gave intermediate fluorescence anisotropy decays in both cases (data not shown).

The rotational correlation time distribution for free riboflavin (Fig. 3B, broken line) shows a main contribution at 0.14 ns which is in agreement with earlier results (van Hoek et al., 1987). When antibody is added, a contribution of ϕ = 1.1 ns and one longer non-resolved correlation time gradually arise, at the expense of the correlation time of free riboflavin (Fig. 3B, solid line). The contribution at 1.1 ns (ϕ_{int}) is due to the restricted motion of flavin and reflects its flexible binding in the antigen binding site. Fab segmental flexibility and tumbling of the whole antibody are apparently slower than the timescale of observation (Hanson et al., 1981; Holowka and Cathou, 1976) and therefore result in a contribution at infinite time (ϕ_∞).

The rotational correlation time distribution pattern of free 1,5-dihydroriboflavin is more complex than that of riboflavin

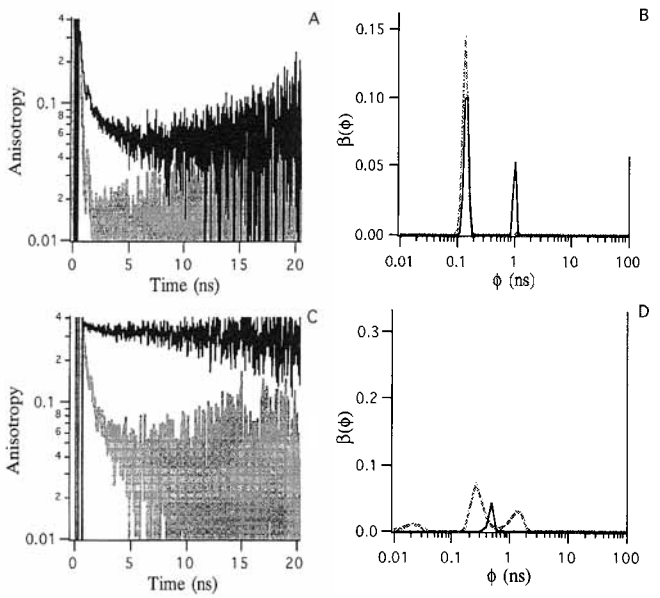


Fig. 3. Experimental fluorescence anisotropy decays and rotational correlation time distributions of free and αRfmodG$_1$-bound riboflavin and 1,5-dihydroriboflavin. The broken curves reflect free riboflavin (A, B) or free 1,5-dihydroriboflavin (C, D). The solid curves reflect bound species in all cases. In all experiments the residuals of the fit were randomly scattered around zero, indicating an optimal fit. The spike at 100 ns corresponds to the infinite anisotropy and is referred to as ϕ_∞. For both free riboflavin and free 1,5-dihydroriboflavin no ϕ_∞ was observed.

(Fig. 3D, broken line). There is a main contribution at 0.3 ns (58%) which is in fair agreement with the value obtained for free reduced flavin mononucleotide (FMN; Leenders et al., 1993). The other contributions are ascribed to self-association of 1,5-dihydroriboflavin molecules, since at lower 1,5-dihydroriboflavin concentrations the distribution pattern approaches that of free reduced FMN which is much more soluble in water.

Upon addition of increasing amounts of antibody, the rotational correlation time spectra gradually change from the initial free to the final bound situation represented by the curves in Fig. 3D. The peak at 0.4 ns (ϕ_{int}) reflects rapid internal motion of the flavin in the antigen binding site and the contribution at ϕ_∞ is again the result of a superposition of Fab segmental flexibility and tumbling of the whole antibody. Inspection of Fig. 3 B and D reveals that at the highest antibody concentration free riboflavin is still present, whereas all 1,5-dihydroriboflavin has been bound.

For the IgM αRfmodM, we obtained basically similar results, but a relatively higher antibody concentration was needed to bind all the 1,5-dihydroriboflavin and it was not possible to bind all the riboflavin.

The rotational correlation time distribution of antibody-bound flavin provides information about the motional freedom of riboflavin in the antigen binding site. From the relative contribution of the internal motion (ϕ_{int}) with respect to ϕ_∞ one can derive the so-called order parameter S, defining the angular displacement ψ of the riboflavin in the antigen binding site. The internal rotational correlation time ϕ_{int} and the order parameter S define the diffusion constant D_1 of this reoriental motion (see Experimental Procedures). These parameters are collected in Table 2 for riboflavin and 1,5-dihydroriboflavin bound to both αRfmodG$_1$ and αRfmodM. From the order parameter S, it is clear that the motion of 1,5-dihydroriboflavin bound to αRfmodG$_1$ is more restricted than that of flexibly bound riboflavin.

Table 2. Geometric and dynamic analysis of fluorescence anisotropy of antibody-bound riboflavin and 1,5-dihydroriboflavin.

Antibody	Antigen	S	ψ	ϕ_{int}	$D_{\perp}$
			deg.	ns	ns^{-1}
αRfmodG$_1$	riboflavin	0.59	62	1.1	0.10
αRfmodG$_1$	1,5-dihydroriboflavin	0.93	24	0.5	0.05
αRfmodM	riboflavin	0.70	54	1.4	0.06
αRfmodM	1,5-dihydroriboflavin	0.77	45	1.1	0.06

Table 3. Dissociation constants for binding of αRfmodM and αRfmodG$_1$ to riboflavin and 1,5-dihydroriboflavin.

Antibody	K_d for	
	riboflavin	1,5-dihydroriboflavin
	μM	
αRfmodM	27	16
αRfmodG$_1$	5.8	0.071

In addition, the motional dynamics of αRfmodG$_1$-bound riboflavin are larger than for 1,5-dihydroriboflavin (reflected in $D_{\perp}$, see Table 2). In contrast, the order parameters for riboflavin and 1,5-dihydroriboflavin when bound to αRfmodM reveal that in this case there is only a slight difference in the flexibility of binding.

Determination of affinity constants. The slow anisotropy decay of 1,5-dihydroriboflavin in the presence of αRfmodG$_1$ (Fig. 3C) strongly suggests that all reduced riboflavin is bound. The fluorescence anisotropy decay of 1,5-dihydroriboflavin in the presence of αRfmodM is the same, indicating that here too all 1,5-dihydroriboflavin is bound. This implies that we can assign curves corresponding to free and completely bound reduced riboflavin and therefore we are able to calculate the fractions of bound and unbound flavin using fractional analysis (Pap et al., 1995). The reduced χ^2 values obtained from these analyses indicate a very good quality of the fits ($0.99 < \chi^2 < 1.07$ for αRfmodG$_1$ and $0.99 < \chi^2 < 1.41$ for αRfmodM).

For riboflavin we could not define a population in which all the flavin was bound, impeding fractional analysis. The rotational correlation time spectra of free and bound riboflavin, however, are less complicated and it is therefore possible to use the relative contribution of free riboflavin as an extent of the fraction of free riboflavin present in the solution (Fig. 3B for αRfmodG$_1$). The contribution of the rotational correlation time at 0.14 ns was normalized to the initial anisotropy of 0.38 (Leenders et al., 1990). This approach allows the determination of affinity constants for weakly bound riboflavin, circumventing the problem that saturation of binding sites could not be reached.

The dissociation constants are calculated according to Eqn (7) (Table 3). Since the error in these values is estimated to be less than 10% we have indeed succeeded in obtaining antibodies with a higher affinity for 1,5-dihydroriboflavin.

DISCUSSION

The fluorescence decay characteristics of free and αRfmodG$_1$-bound 1,5-dihydroriboflavin hardly differ. This is remarkable because the majority of the 1,5-dihydroriboflavin is bound, indicating that the local environment of 1,5-dihydroriboflavin when bound is not very different from that in aqueous solution.

From MEM analysis of the fluorescence of 1,5-dihydroriboflavin and riboflavin in binding equilibrium with both investigated antibodies, two rotational correlation times, a short ϕ_{int} and a long ϕ_{∞}, have been obtained. Because of the relatively short fluorescence lifetimes of riboflavin and 1,5-dihydroriboflavin, it is not possible to determine the exact origin of ϕ_{∞}. Previously reported studies, however, on depolarizing motions of dansyl bound to IgM and IgG antibodies, ascribe this motion to wobbling of the Fab subunits (segmental flexibility) rather than tumbling of the whole antibody molecule (Hanson et al., 1981; Holowka and Cathou, 1976). Moreover, this global tumbling should have little influence on the decay of anisotropy if the rate of intramolecular flexible motion is significantly larger than that of overall tumbling and if this motion occurs over a sufficiently angular range to depolarize light considerably.

Comparison of the fluorescence anisotropy decays of αRfmodG$_1$-bound riboflavin and 1,5-dihydroriboflavin already suggests that at an antibody concentration where 1,5-dihydroriboflavin is clearly totally immobilized, riboflavin is only partly bound. Further analysis reveals that both αRfmodG$_1$ and αRfmodM have a higher affinity for 1,5-dihydroriboflavin than for riboflavin. Comparison of the affinities of both antibodies for 1,5-dihydroriboflavin shows an overall higher affinity of the IgG$_1$ αRfmodG$_1$ for both riboflavin forms. The affinity of αRfmodG$_1$ for 1,5-dihydroriboflavin is typical for antibodies after the second immune response. The affinities of the IgM αRfmodM for both flavin forms are low and typical for IgM antibodies that generally have low affinity for the antigen since they are formed prior to affinity maturation. From the dynamical and geometrical parameters, we conclude that binding of 1,5-dihydroriboflavin to αRfmodG$_1$ is less flexible than binding of riboflavin so that not only the affinity for this reduced flavin form is higher but also the specificity. For the IgM this difference in flexibility of reduced or oxidized flavin binding is less evident. When we consider the IgM as a product of the primary immune response and the IgG$_1$ as a product of the secondary response, these results show that during affinity maturation not only the affinity for 1,5-dihydroriboflavin increases, but also the specificity of binding to this molecule is strongly improved.

In ELISA experiments (results not shown) we can not distinguish between binding to the riboflavin and the *N*(5)-benzoyl-*N*(10)-ribitylsuccinimide ester flavin that served as hapten and represents the riboflavohydroquinone form. This is not surprising because, although a powerful method, limitations and artefacts have been reported for ELISA (Goldberg and Djavadi-Ohaniance, 1993; Pesce and Michael, 1992; Schwab and Bosshard, 1992). It is not only the fact of being unable to distinguish between both riboflavin forms which is a drawback of using the ELISA method for this system but, in case of the IgM, it is almost impossible to determine the generally weak affinities of such large, multivalent molecules on a surface. Time-resolved fluorescence allows us to obtain quantitative information on the binding of both riboflavin an 1,5-dihydroriboflavin to the antibody molecule.

CONCLUSION

Time-resolved polarized fluorescence spectroscopy proves to be an excellent technique to study binding of riboflavin and, in particular, flavohydroquinone to these antibodies. This method not only allows us to determine the affinity for both flavin forms but also provides information about the specificity and flexibility of the binding. Moreover, because of the high dynamic range,

weak affinities can also be determined from analysis of rotational motion.

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