Cofactor-containing antibodies: Crystal structure of the original yellow antibody

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Antibodies are generally thought to be a class of proteins that function without the use of cofactors. However, it is not widely appreciated that antibodies are believed to be the major carrier protein in human circulation for the important riboflavin cofactor that is involved in a host of biological phenomena. A further link between riboflavin and antibodies was discovered 30 years ago when a bright-yellow antibody, IgGGAR, was purified from a patient with multiple myeloma who had turned yellow during the course of her disease. It was subsequently shown that the yellow color of this antibody was due to riboflavin binding. However, it was not known how and where riboflavin was bound to this antibody. We now report the crystal structure of this historically important IgGGAR Fab at 3.0-Å resolution. The riboflavin is located in the antigen-combining site with its isoalloxazine ring stacked between the parallel aromatic moieties of TyrH33, PheH58, and TyrH100A. Together with additional hydrogen bonds, these interactions reveal the structural basis for high-affinity riboflavin binding. The ligand specificity of IgGGAR is compared with another riboflavin-binding antibody, IgGDOT, which was purified from a second patient with multiple myeloma. The crystal structure of IgGGAR provides a starting point for attempts to understand the physiological relevance and chemical functions of cofactor-containing antibodies.

A bright-yellow antibody, IgGGAR, from a patient with multiple myeloma, was discovered and characterized by Farhangi and Osserman in 1976 (1). The patient ("Gar") was referred to the Francis Delafield Hospital, New York, in May 1973, with a diagnosis of multiple myeloma. Physical examination confirmed that the patient presented with an intense and unusually bright-yellow coloration of the skin (xanthoderma) and hair (xanthotrichia), but normal white sclera, suggesting a non-bilirubin-related abnormality. The cause of pigmentation was bright yellow, and all the xanthochromia was associated with a monoclonal antibody, IgGGAR. The patient with multiple myeloma who had turned yellow during the course of her disease. It was subsequently shown that the yellow color of this antibody was due to riboflavin binding. However, it was not known how and where riboflavin was bound to this antibody. We now report the crystal structure of this historically important IgGGAR Fab at 3.0-Å resolution. The riboflavin is located in the antigen-combining site with its isoalloxazine ring stacked between the parallel aromatic moieties of TyrH33, PheH58, and TyrH100A. Together with additional hydrogen bonds, these interactions reveal the structural basis for high-affinity riboflavin binding. The ligand specificity of IgGGAR is compared with another riboflavin-binding antibody, IgGDOT, which was purified from a second patient with multiple myeloma. The crystal structure of IgGGAR provides a starting point for attempts to understand the physiological relevance and chemical functions of cofactor-containing antibodies.

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Abbreviations: CDR, complementarity-determining region; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

Data deposition: Atomic coordinates and diffraction data for IgGGAR Fab have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2FL5).

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Upon binding of riboflavin, 258 Å² of the molecular surface of ribityl side-chain extends out to the pocket rim (Figs. 2 and 3). Buried in the narrow slot in the binding pocket, whereas the trapped in the binding site such that the isoalloxazine ring is occluded by the residues TyrH33, PheH58, TyrH100A, and AsnH50, according to the Kabat–Wu database (11), the key residues for interactions with GlyL95, TyrL95A, ProL96, and ValH95. Accordingly, the isoalloxazine ring also makes van der Waals’ interactions and four hydrogen bonds are made between antibody and riboflavin, with only 17 of these van der Waals’ interactions from the light chain. Residues from four CDR loops contact the riboflavin: GlyL95 (L3), TyrL95A (L3), ProL96 (L3), TyrH33 (H1), AsnH50 (H2), ArgH52 (H2), GluH56 (H2), PheH58 (H2), ValH95 (H3), and TyrH100A (H3). No contacts are made with CDR loops L1 and L2, although the latter is not surprising, because most haptns are unable to access CDR L2 when bound in the combining site (10).

Binding of riboflavin occurs in a narrow cleft, with the vitamin isoalloxazine ring stacked between the parallel aromatic groups of TyrH33 (with the si-face of riboflavin), PheH58 (si-face), and TyrH100A (re-face), with distances between the isoalloxazine ring and the respective aromatic rings from these three residues of 3.2, 3.5, and 3.4 Å (Figs. 3A and 4). These π–π stackings presumably contribute to the high-affinity binding of riboflavin.

The isoalloxazine ring of flavins is amphipathic, because the xylene portion is hydrophobic, and the pyrimidine moiety is hydrophilic. The isoalloxazine ring of flavins in IgGGAR molecules C and D hydrogen bonds to O5' of riboflavins bound to molecules A and B, respectively.

For the ribityl side-chain interactions, two hydrogen bonds are formed between the side chain and the antibody: O2’ to the carboxyl group of GluH56, and O5’ to the guanidinium group of ArgH52 (Figs. 3 and 4). The ribityl moiety also makes van der Waals’ interactions with TyrH33 and PheH58. In the crystallographic asymmetric unit, a side chain from a neighboring molecule hydrogen bonds with the riboflavin; GluH56 in IgGGAR molecules C and D hydrogen bonds to O5’ of riboflavins bound to molecules A and B, respectively.

The oxidized riboflavin in IgGGAR exhibits a planar isoalloxazine-ring configuration (Figs. 2 and 3). IgGGAR loses its yellow color under reducing conditions (data not shown). Although “butterfly” flavin conformations have been observed in a number of crystal structures under reducing conditions (12), other cases have been observed where the reduced flavin is in an almost planar conformation (13). Planar oxidized or puckered reduced flavins have both been observed in the same active site with no large conformational changes in other systems (12).

However, whether any structural rearrangements of IgGGAR occur in a reducing environment needs further exploration. In this crystal structure, no water-mediated hydrogen bonds are formed between antibody and riboflavin, as seen for some other flavin-protein structures. However, because this structure is at comparatively modest resolution (3 Å), it is difficult to define all of the bound water molecules.

**Structural Basis for Ligand Specificity.** FMN, FAD, and a variety of riboflavin analogues (Fig. 1) bind IgGGAR with various affinities (1, 2, 14). The $K_d$ values for FMN and FAD are 2.2 nM and 8 nM, respectively, as compared with the $K_d$ for riboflavin of 1.8 nM.
These similar $K_d$ values indicate that binding of IgG$^\text{GR}$ with flavins is rather insensitive to the relative size and charge of the substituent at the C5’/H11032 position of the ribityl moiety. FMN, with a negatively charged phosphate group at C5’/H11032, exhibits only a 4-fold reduction in affinity relative to riboflavin, whereas FAD, adenine attached to C5’/H11032 by a phosphodiester linkage binds IgG$^\text{GR}$ with a 10-fold decrease in its $K_d$ relative to riboflavin. These findings correlate with our structural results in that the isoalloxazine ring is clearly the most important determinant for recognition and likely contributes most of the binding energy where the ribityl side chain extends out of the binding site toward the antibody surface.

A series of riboflavin analogues have been tested in binding studies with IgG$^\text{GR}$ (14). Derivatives with large substituent at the 8-position of the flavin, such as roseoflavin and 8-propylaminoriboflavin (Fig. 1), have only an ~10-fold decrease in affinity relative to riboflavin, whereas FAD adenine attached to C5’ by a phosphodiester linkage binds IgG$^\text{GR}$ with a 10-fold decrease in its $K_d$ relative to riboflavin. These findings correlate with our structural results in that the isoalloxazine ring is clearly the most important determinant for recognition and likely contributes most of the binding energy where the ribityl side chain extends out of the binding site toward the antibody surface.

Fig. 3. Stereoview of the IgG$^\text{GR}$ antigen-binding site. (A) The combining site with bound riboflavin. Hydrogen bonds are shown as dotted blue lines. The isoalloxazine ring is $\pi$-stacked between aromatic residues TyrH33, PheH58, and TyrH100A, the N5 atom of the ring hydrogen bonds to AsnH50, and the ribityl side chain contributes two hydrogen bonds to ArgH52 and GluH56. (B and C) Top view (B) and side view (C) of shape complementarity of riboflavin in the antibody IgG$^\text{GR}$-combining site prepared with PyMOL (http://pymol.sourceforge.net). The isoalloxazine ring is trapped in a narrow slot in the combining site, and the ribityl side chain rests on the outer surface of the combining site. The molecular surface is colored by electrostatic potential (calculated with the program APBS (28) with a 1.4-Å probe radius and contoured between $-20$ and $+20$ kT).

Comparison with Other Flavin-Binding Proteins. Flavins can participate in both one- and two-electron transfer processes and play a pivotal role in a host of biological phenomena. These flavoprotein enzymes catalyze a vast number of biochemical reactions, from oxidases to dehydrogenases and monoxygenases (15). The isoalloxazine ring is the redox-active center of the flavin, where its flavin redox potential is regulated by its protein environment. In a typical redox reaction, when electrons are transferred into the isoalloxazine ring through the electron-deficient N5 atom (16), the negative charge is dispersed over C4A, N5, N10, and N1 (17). Stabilization of this negative charge by the protein is an important factor for the redox potential; a positively charged environment around the pyrimidine ring will increase its potential, whereas a negatively charged or hydrophobic environment will decrease it (15). In the available crystal structures of >40
riboflavin might adopt a similar binding mode with IgGDOT as tively), together with the similar affinity for flavins, suggests that light and heavy chains, respec-
only very weakly, with 

flavoproteins, the majority of the flavin–protein interactions are made with the ribitol side chains of riboflavin, FMN, or FAD (18). In IgG\textsuperscript{GAR}, the pyrimidine ring of riboflavin is buried in a mostly hydrophobic environment, where the N5 atom is positioned inside the binding pocket. Thus, N5 could be difficult to access by most large potential redox substrates, but molecular oxygen and its redox derivatives would not be excluded.

IgG\textsuperscript{DOT} is another natural riboflavin-binding antibody that was purified from a patient also with multiple myeloma, xantho-
derma, and xanthatrichia (3). Purified IgG\textsuperscript{DOT} is also bright yellow, and its ligand was identified as riboflavin. IgG\textsuperscript{DOT} shares an almost identical affinity for flavins with IgG\textsuperscript{GAR}, with \( K_d \) values for riboflavin, FMN, and FAD of 1.7, 6.6, and 18 nM, respectively (2). Comparison of their amino acid sequences (2) indicates that the key residues for riboflavin binding in IgG\textsuperscript{GAR}, TyrL95A, TyrH33, and TyrH100A, are conserved in both IgG\textsuperscript{GAR} and IgG\textsuperscript{DOT} antibodies. The sequence similarity (51% and 45% identity between the light and heavy chains, respec-
tively), together with the similar affinity for flavins, suggests that riboflavin might adopt a similar binding mode with IgG\textsuperscript{DOT} as with IgG\textsuperscript{GAR}. It is noticeable, however, that some other key binding residues in IgG\textsuperscript{GAR}, such as AsnH50, ArgH52, and PheH58, are not conserved and are substituted by IleH50, AsnH52, and SerH58, respectively, in IgG\textsuperscript{DOT}, suggesting a slightly different or modified flavin-binding mode.

**Discussion.** Immunoglobulins and albumin are the main riboflavin-carrier proteins in the plasma (19). Albumin binds riboflavin only very weakly, with \( K_d \) values of 3.8 to 10.4 mM (19), whereas high-affinity binding was detected in the normal human plasma Ig fraction with \( K_d \) values of 2.43 and 0.07 mM for two proposed binding sites (20). Any assumptions about the physiological role of riboflavin-binding immunoglobulins must center on whether one considers the bound riboflavin an antigen or whether the riboflavin-binding myeloma proteins simply reflect clonal expansion during disease of that subclass of antibodies that normally bind riboflavin. If, in our studies, riboflavin had been found to bind outside of the combining site, one might have simply concluded that, although it is curious that immunoglobul-

![Fig. 4. Schematic presentation of riboflavin binding site in IgG\textsuperscript{GAR}. Residues forming van der Waals’ interactions with the riboflavin are indicated by an arc with radiating spokes toward the ligand atoms they contact; those participating in the hydrogen bonds with the riboflavin are shown in ball-and-stick representation. Hydrogen bonds are illustrated as blue dotted lines. Carbon atoms are colored in black, nitrogen atoms in blue, and oxygen atoms in red. Atom names of the riboflavin are labeled. The figure was generated from the program \textsc{sig} \textsc{lo}t (29).](image-url)
complex redox reactions that destroy bound antigens (data not shown).

Materials and Methods

Purification, Crystallization, and Data Collection. Human monoclonal antibody IgG\textsubscript{GAR} Fab was produced by standard protocols. The yellow native IgG\textsubscript{GAR} was purified from material received from the late Elliott Osserman (Columbia University, New York). IgG\textsubscript{GAR}, a human IgG2(\lambda) Ig, was isolated from an 80-year-old patient, as reported in ref. 1. In brief, IgG\textsubscript{GAR} was recovered from the patient’s plasma by ammonium sulfate precipitation, followed by gel filtration, as described in ref. 21. The purified IgG from the 30-year-old frozen sample was digested to Fab fragments with 1% (wt/wt) papain for 20 h at 37°C, followed by size-exclusion (Superdex-200 column; Amersham Pharmacia) and protein A-affinity chromatography.

IgG\textsubscript{GAR} Fab was concentrated to 10.7 mg/ml in 0.025 M Tris (pH 7.5) and 100 mM NaCl. Yellow Fab crystals were grown by the sitting-drop vapor-diffusion method from 23% (wt/vol) PEG 4000, 0.2 M diammmonium hydrogen citrate, and 0.05 M Tris (pH 7.5). A 3.0-Å data set was collected at the Stanford Synchrotron Radiation Laboratory (SSRL) on beamline 11–1 [Area Detection Systems Corporation (Poway, CA) Quantum 315 charge-coupled device detector] from a single crystal with 25% (vol/vol) glycerol as cryoprotectant. The crystal space group is P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, with four Fab/riboflavin complexes per asymmetric unit ($V_m = 2.8 \text{Å}^3$/Da, 57% solvent). Data were integrated and scaled with HKL2000 (22) (see Table 1).

Structure Determination. The IgG\textsubscript{GAR} Fab structure was determined by molecular replacement methods by using the program MOLREP (23). A Fab fragment from human Ig H\textsubscript{H} (IgG1, \lambda) (PDB code 8fab; molecule 2) was used as the search model for rotation and translation function calculations (correlation coefficient = 0.38, $R_{crys} = 0.54$ for the resolution range 40.0–4.0 Å). A pseudotranslation relates pairs of Fabs by the vector (0.33, 0.5, 0) or by a/3 + b/2, as deduced from native Patterson maps. Structural refinement was completed to 3.0-Å resolution by using the program CNS (24) to a final $R_{crys} = 0.24$ and $R_{free} = 0.29$ for all data (see Table 1). Model rebuilding was performed by using the program O (25). Data collection and refinement statistics are summarized in Table 1.

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