Flavoproteins are an important and widespread class of enzymes that generally catalyze redox reactions. All flavoproteins include a covalently or noncovalently bound flavin; the two most common are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Especially interesting is that although they catalyze a wide variety of reactions, all flavoproteins have an identical isoalloxazine ring system at the active site (Fig. 1). The ring system can be tuned to change its redox potential and reactivity by the protein microenvironment of the flavin binding site. Small changes in the properties of the protein allow this ring system to display a range of redox potentials and to be involved in the catalysis of many different types of reactions.

An especially successful method for probing the mechanisms by which the protein environment modulates the reactivity of the flavin is the use of modified flavins to assess the interactions at specific positions of the flavin with the protein atoms surrounding the flavin (reviewed in Ref. 1). This method often requires that the flavin be removed from a flavoprotein and then replaced with a chemically modified flavin. Aspects of the flavin microenvironment that can be studied include the relative distance between particular atoms in the flavin and the protein, the solvent accessibility of regions of the isoalloxazine ring, and the nature of the groups (i.e. positively charged, hydrogen bonding, neutral, etc.) surrounding the flavin. The first characteristic can be addressed by studying a series of flavins modified at a particular position by substituents of varying size. The larger the size of the modified group that still allows flavin binding, the larger the distance between flavin and protein is proposed to be. Solvent accessibility can be studied by placing a reactive group (often photoactivatable) on the flavin and determining whether it reacts with protein residues and/or reactive species added to the solvent (2–7). Reactivity with protein residues over the solvent-borne species suggests close proximity to the protein, whereas preferential reactivity with solvent-borne species suggests that the position is solvent-accessible. To determine the chemical nature of amino acid residues in the flavin binding site, modified flavins can be prepared, and the changes in their absorption spectra upon protein binding can be analyzed (7–10).

Other methods that have been used to investigate the environments of flavins in flavoproteins are 13C and 15N NMR spectroscopy (11–13). Chemical shifts from free flavin in aqueous and nonpolar systems can be compared with the chemical shifts from flavin bound to apoprotein. For example, finding that the chemical shifts in protein-bound flavin are similar to those found in the aqueous flavin suggests that the atom being probed in the flavin is in a polar environment in the protein. These methods can also give information about the involvement of flavin atoms in hydrogen bonding and the proximity of atoms to charged groups in the protein.

Several flavoproteins have been used for many of the studies, mainly because it has been possible to obtain large quantities of protein and because of the ease with which the flavin can be removed and then replaced without damage to the apoprotein. Among these is the first known flavoprotein, old yellow enzyme (OYE), which has been used in the majority of studies involving modified flavins (see Ref. 1 for a review). OYE still has no known physiological function, although recent work suggests that it may be involved in the NADPH-dependent reduction of enones to ketones (14). In addition to the modified flavin studies, detailed 13C and 15N NMR studies have been performed on the FMN bound to OYE (11–13). Together, both sets of studies led to a tentative active site structure for the enzyme (1).

The crystal structure of the OYE1 isozyme from Saccharomyces carlsbergensis was solved at 2.0 Å resolution (15) and shows that the overall structure of OYE1 is an α/β barrel. The flavin binds to the protein at the C-terminal end of the barrel, with the isoalloxazine ring approximately perpendicular to the barrel axis. At this resolution, all of the amino acid residues that interact with the flavin and ordered solvent molecules are clearly visible, allowing us to compare the crystallographically determined structure of the OYE1 active site to that proposed...

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¶ The abbreviations used are: FMN, flavin mononucleotide; OYE, old yellow enzyme.
by NMR and modified flavin experiments. This, in turn, will allow us to assess the utility of these methods for elucidating the true microenvironment of flavins in flavoproteins. Because these experiments are still widely used to study flavoproteins for which no three-dimensional structure is known, it is important to understand how the results from spectroscopy compare with those obtained by crystallography.

**EXPERIMENTAL PROCEDURES**

The structure of oxidized OYE used for this analysis was derived from the published 2.0 Å resolution structure of OYE1 from S. carlsbergensis (Ref. 15; PDB2 1OYA) after further partial refinement at 1.65 Å resolution. This model contains 428 solvent molecules, a chloride ion, and a magnesium ion in addition to all the protein and flavin atoms and has an R-factor and R_{free} from 10 to 1.65 Å of 19.1% and 25.0%, respectively. Refinement is continuing, and a description of the fully refined structure will be published separately. The model of the reduced enzyme is from the original 2.0 Å resolution refinement (Ref. 15; PDB 1OYB). To analyze the flavin interactions, the structure was examined using the CHAIN program (16) on a SGI Indigo2 workstation equipped with a CrystalEyes2 stereo viewing system.

**RESULTS**

A summary of the features of the flavin environment inferred from the NMR and modified flavin studies is presented in Table I, along with the results from the crystallographic studies. These results are more fully described in the following sections. As one caveat, we note that the OYE used for the artificial flavin and spectroscopic studies has been discovered to be a mixture of two OYE isozymes found in S. carlsbergensis, but only one of these, OYE1, is of known sequence (17). A comparison with the sequences of the two isozymes (OYE2 and OYE3) from S. cerevisiae (18, 19) reveals that 21 of the 22 amino acids that have atoms within 5.0 Å of the flavin in the OYE1 structure are completely conserved in the three isozymes (OYE1 from S. carlsbergensis and OYE2 and OYE3 from S. cerevisiae). The exception is OYE1 Phe-296, which is changed to a serine in OYE2 and OYE3. This phenylalanine residue is part of a mobile loop that approaches (3.9 Å) but does not pack tightly against the dimethylbenzene moiety. Based on this high similarity between the isozymes from two different yeast strains, we assume that the flavin environment in the other isozyme from S. carlsbergensis will also be highly conserved, so that our analysis based on the structure of OYE1 is valid. Support for this assumption comes from the observation that none of the published spectroscopic and artificial flavin studies mentioned heterogeneous behavior such as would be expected if the two isozymes had significant differences in the flavin environment. A second caveat is that it must be recalled that the crystal structure is an average conformation of the protein and does not account for its conformational fluctuations.

**General Flavin Environment**—Spectroscopic studies with modified flavins have predicted that the flavin in OYE is generally accessible to solvent. This was especially evident along the dimethylbenzyl edge of the flavin. Although the pyrazine and pyrimidine rings were also shown to be partly solvent-accessible, the atoms within these rings were predicted to be closely associated with the protein (5). The tight interaction with the protein is also evidenced by the flavin binding constant of $10^{-10}$ M (20).

As previously stated, the crystal structure reveals a long solvent channel through the loops at the C-terminal end of the α/β barrel, which results in exposure of the si face of the flavin to solvent. The re face, on the other hand, is completely buried by interactions with protein main chain and side chain groups. The dimethylbenzyl ring of the flavin is solvent-accessible both from the si face and along the edge, whereas the other two rings are accessible only from the si face.

**Interactions in the Dimethylbenzene Ring of FMN**—Studies of flavins modified at C6 (5, 6) and C8 (2, 8) showed the dimethylbenzene ring (containing atoms C6, C7, C7A, C8, C8A, and C9) to be solvent-accessible. NMR studies supported this by showing that C9 behaves as if it is in a polar environment (12). Both of these conclusions are supported by crystallography, which shows that C7, C8, and C9 are greater than 3.5 Å away from any protein atom and are exposed to bulk solvent.

The results of modified flavin studies also suggested that there could be a positive charge near C6 and a bound water molecule near C8. These conclusions were not supported or refuted by the NMR studies, because C6 was not studied, and the resonance for C8 was not identified. The crystallographic results show that there are no positive charges near C6; in fact, the only atoms less than 3.5 Å away from C6 are a main chain oxygen and nitrogen. There is also no crystallographic evidence

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**Table I**

| Flavin position | Predicted interaction from NMR studies | Predicted interaction from modified flavin studies | Crystallographically determined interaction | Atoms within 3.5 Å of this position |
|----------------|----------------------------------------|-----------------------------------------------|---------------------------------------------|----------------------------------|
| N1             | Protected from solvent, no H-bonds     | Positive charge nearby                         | Protected from solvent, no H-bonds, positively charged Arg nearby | Arg-243 N; Aan-194 N; Pro-35 O; Cl ; Pro-35 C; Arg-243 N; His-191 (N, Cc); Gln-114 Ne |
| C2 and O2A     | O2A is H-bonded                        | Protected from solvent, positive charge nearby | Solvent-accessible from above, hydrogen bond from protein to O2, positively charged Arg nearby | Pro-350; Cl ; Cl ; Thr-37 (O,N, Os); Gly-72N; Thr-37N |
| N3             | H-bond to protein/and polarization of C4-O4A and C2-O2A carboxyls | H-bond to protein                             | H-bond to protein                           | Gln-114 Oe; His-191 Ne; Cl |
| C4 and O4A     | O4A is H-bonded                        | Limited solvent accessibility, thiol nearby which donates an H-bond to O4A | Solvent-accessible from above, H-bond from protein to O4, no thiol | Pro-350; Cl ; Cl ; Thr-37 (O,N) |
| N5             | Strong hydrogen bond or positive charge nearby | Positive charge nearby                         | Hydrogen bond from protein, no positive charge | Thr-37 (O,N) |
| C6             | NS"                                | Solvent-accessible, positive charge nearby | Solvent-accessible, no charged residues nearby | None |
| C7 and C7A     | Polar environment                      | Solvent-accessible                            | Solvent-exposed                            | None |
| C8 and C8A     | Polar environment                      | Solvent-exposed, close to a bound water molecule | None |
| C9 and C9A     | Polar environment                      | NS                                             | Solvent-exposed                            | None |
| N10            | Polar environment                      | NS                                             | Solvent-exposed from above                 | None |

"NS, not studied.
for a discretely bound water near C8, although the atom is certainly exposed to bulk solvent.

**Interactions in the Pyrimidine Ring of FMN**—The results of modified flavin studies suggested that this portion of the isoalloxazine ring is somewhat accessible to solvent but is protected along the edges. Specifically, C2 was shown to be protected from solvent, and there was evidence of a positive charge in the N1-C2 region (7, 10). N3 was hypothesized to be involved in a hydrogen bond with a protein atom, and C4 was suggested to be accessible to solvent, with O4A weakly hydrogen bonded to a thiol in the flavin binding site (21). The presence of a thiol in the C4-O4A region was later questioned because the sequence of OYE1 contains no cysteine residues (17); however, it was still considered possible because the artificial flavin studies were performed with a mixture of isozymes from *S. carlsbergensis*, one of which could contain a cysteine residue (18). NMR studies supported the hydrogen bonds at N3 and O4A, although it was suggested that the shifts at N3 could also be explained by polarization of the C4-O4A and C2-O2A carbonyls (11, 12). In addition, the NMR studies suggested that N1 was protected from solvent and was not involved in any hydrogen bonds, and that O2A was involved in a hydrogen bond with the protein.

Crystallographic studies support the NMR results at N1, showing that this atom is protected from solvent and is not involved in hydrogen bonds (Fig. 1). Although there are some possible hydrogen bond donors within 3.5 Å of N1, these groups are already involved in more favorable hydrogen bonds with other atoms. At atoms C2/O2A, the results from NMR are again supported, showing that O2A is involved in two strong hydrogen bonds with Gln-114 Ne and Arg-243 Nζ. The modified flavin studies are also supported, because C2 is only accessible via the *si* face, and Arg-243 accounts for the predicted positive charge nearby. The results at N3 are also borne out by crystallography in the form of a hydrogen bond to Gln-114 Oe. It is at C4 where there are major differences between the modified flavin results and the crystal structure. The structure shows that O4A is involved in strong hydrogen bonds with Thr-37 Oγ and the main chain nitrogen of Gly-72, but not with a thiol. In addition, C4 is not particularly exposed to solvent, because the hydrogen bonds prevent access to this atom, except from the *si* face of the flavin. Its solvent accessibility is similar to that of C2.

**Interactions in the Pyrazine Ring of FMN**—This central flavin ring only contains two atoms that have not already been discussed, N5 and N10. NMR studies supported the hydrogen bonds at N3 and O4A, although it was suggested that the shifts at N3 could also be explained by polarization of the C4-O4A and C2-O2A carbonyls (11, 12). In addition, the NMR studies suggested that N1 was protected from solvent and was not involved in any hydrogen bonds, and that O2A was involved in a hydrogen bond with the protein.

Crystallographic studies support the NMR results at N1, showing that this atom is protected from solvent and is not involved in hydrogen bonds (Fig. 1). Although there are some possible hydrogen bond donors within 3.5 Å of N1, these groups are already involved in more favorable hydrogen bonds with other atoms. At atoms C2/O2A, the results from NMR are again supported, showing that O2A is involved in two strong hydrogen bonds with Gln-114 Ne and Arg-243 Nζ. The modified flavin studies are also supported, because C2 is only accessible via the *si* face, and Arg-243 accounts for the predicted positive charge nearby. The results at N3 are also borne out by crystallography in the form of a hydrogen bond to Gln-114 Oe. It is at C4 where there are major differences between the modified flavin results and the crystal structure. The structure shows that O4A is involved in strong hydrogen bonds with Thr-37 Oγ and the main chain nitrogen of Gly-72, but not with a thiol. In addition, C4 is not particularly exposed to solvent, because the hydrogen bonds prevent access to this atom, except from the *si* face of the flavin. Its solvent accessibility is similar to that of C2.

**Fig. 1. Flavin environment diagram.** The protein environment around the flavin is shown with the FMN in bold lines, and the protein amino acid residues are represented by their one-letter code and their number in the sequence. Only the portion of the side chain that interacts directly with the FMN is shown. Hydrogen bonds between flavin atoms and protein atoms are indicated by dotted lines, and the bond distance is given in angstroms nearby. The numbering scheme for the isoalloxazine ring is given with small labels in italics.
chain nitrogen of Thr-37 to N5 (Fig. 1).

**Geometry at N5 and N10**—The geometry of the isoalloxazine ring in both the reduced and oxidized states in OYE was analyzed by NMR spectroscopy. Experiments with the oxidized enzyme suggested that N10 is partly sp² hybridized in the oxidized enzyme, whereas N5 has the expected sp³ character. In the reduced enzyme, N10 behaved as if it took on more sp² character, whereas N5 became nearly fully sp³ in character (11, 12). It is clear that the crystal structure is consistent with the NMR results at N10 and N5 in both redox states. In the oxidized enzyme, N10 is partly tetrahedral because it lies 0.08 Å above the plane defined by the three atoms connected directly to it (C1R, C9A, and C5A). In the reduced enzyme, this distance decreases to 0.04 Å, consistent with a more sp² character but also possibly influenced by the lower resolution of the refinement of the reduced form. It is more difficult to assess the situation at N5, because it is only covalently bonded to two non-hydrogen atoms (C4A and C5A). All that can be said is that it lies in the plane of the flavin in the oxidized structure; however, in the structure of reduced OYE, it is puckered significantly out of the plane (15). This puckering may be influenced by the presence of a strong hydrogen bond to the N5 from the Thr-37 main chain nitrogen (Fig. 1). This suggests that N5 has a high degree of sp³ character in the reduced structure.

**DISCUSSION**

**Strengths of NMR and Modified Flavin Studies for Determining Flavin Interactions and Geometry**

NMR spectroscopy is an excellent method to probe the hydrogen bonding interactions of the flavin. Each time a hydrogen bond was proposed by NMR, such an interaction was found in the crystal structure (Table I). Also, no hydrogen bonds that had not been suggested by NMR were identified in the crystal structure. NMR was also a sensitive probe of the environment of each atom, correctly predicting when atoms were protected from or exposed to solvent (Table I). In addition, NMR data could be used to predict the geometry at each of the atoms in the isoalloxazine ring.

Data from modified flavin studies were extremely useful for determining whether a site on the flavin was interacting tightly with the protein, was somewhat more distant, or was completely exposed. It was also possible to determine with accuracy whether or not a particular position was exposed to the solvent. These studies were also useful for predicting hydrogen bonding interactions, especially when used in combination with NMR data.

**Discrepancies between Spectroscopic and Crystallographic Results**

The main problems encountered with both modified flavin and NMR studies occur when the results are extrapolated to make specific predictions of the chemical nature of amino acid residues involved in interactions with the flavins. Conclusions of this type were not ventured in the NMR literature for OYE, but they were proposed for the modified flavin studies. In some cases (those for atoms N1, C2, and N3), the type of interaction predicted is consistent with that observed in the crystal structure (Table I). In the other cases (those for atoms C8, C6, N5, and O4A), the specific prediction did not agree well with the structural observations.

These discrepancies do not appear to be related to dynamic properties of OYE that are not captured in the crystal structure. Instead, it is apparent from examining the reasoning behind the conclusions from modified flavin studies that the discrepancies can often be explained by protein stabilization of particular flavin resonance structures that allow the chemical environment of one region of the flavin to influence the properties of another region of the flavin. The major contributors to oxidized flavin resonance, as determined by NMR (9), are shown in Fig. 2. Because both O2A and O4A are involved in strong hydrogen bonds to protein atoms in OYE (Fig. 1), it is expected that the resonance structures with negative charges at these oxygens (structures B-H) will be stabilized when FMN is bound to OYE. This, in turn, stabilizes partial positive charges at C6, C8, and N10, resulting in a partially polarized flavin, with the pyrimidine ring carrying more negative charge, and the edge of the flavin from C6 to N10 carrying more positive charge. In the following paragraphs, we discuss reasons for the apparent discrepancies between spectroscopic and crystallographic results.

**C8, C8A**—Several lines of evidence from the modified flavin work clearly showed that these atoms are solvent-exposed, and some information was interpreted to indicate a discretely bound water molecule near C8. One reason for assuming solvent accessibility was the decreased reactivity of 8-azido-FMN with protein residues, as compared with other proteins, upon irradiation of protein-bound flavin (2). The authors suggested that this was due to nearby solvent molecules that reacted with the 8-azido group, preventing it from further reaction with the protein. The evidence for the presence of a bound water was the observation that protein-bound 8-fluoro-FMN was not reactive via nucleophilic aromatic substitution by a protein atom but was instead hydrolyzed slowly to 8-hydroxy-FMN, whereas free 8-fluoro-FMN was stable in solution (1). This was interpreted to mean that a bound water molecule was available nearby and reacted preferentially with the 8-fluoro group.

Because it is clear from the crystal structure that whereas C8 is solvent-exposed, there is no discretely bound water molecule near C8, several possibilities are available to explain the modified flavin data. The simplest possibility is that the low reactivity of 8-azido-FMN is due to the fact that there are no protein atoms near C8. The closest atoms are 3.5–5.0 Å away and are poor nucleophiles, such as aliphatic and aromatic carbon atoms. Hence, there is little chance for reactivity with the protein. This leaves bulk water, which is abundant in the system, as the most likely nucleophile. However, this does not explain the increased reactivity of 8-F-FMN, because the 8 position could not be more exposed in protein-bound flavin than in free flavin. Both of these phenomena may be explained by the stabilization of the polarized flavin that results in a partial positive charge on C8. When bound to OYE, with its capacity to stabilize flavins with a negative charge in the N1-C2 locus, the azide moiety in 8-azido-FMN is actually stabilized by the presence of the partial positive charge (Fig. 3a), making it less reactive than an azido group attached to an unpolarized FMN. The rate at which 8-fluoro-FMN undergoes nucleophilic substitution at C8 would be enhanced by the polarized flavin, because the entire dimethylbenzene ring becomes more electron-deficient and therefore more reactive with a nucleophile.

**C6**—The major difference between predictions of modified flavin studies and the crystal structure at C6 is the hypothesis that a positively charged residue exists nearby (6). Because the nearest positively charged atom is 7.5 Å away, the results leading to this hypothesis must be explained in some other way. The evidence for the presence of a positive charge was twofold: (a) a nearby protein group could react with C6 in 6-azido-FMN to form a stable complex, and this was suggested to be an amino group; and (b) a nearby positive charge was postulated to explain the stabilization of the 6-sulfenato-FMN over the 6-sulfoxo-FMN species (Fig. 3b), which is generated when 6-thio-FMN is oxidized in the presence of peroxides (6). Because amino groups are generally positively charged when
present on proteins, such a group nicely explained both of these results.

The only amino acid atom visible in the crystal structure that would be likely to react with C6 of 6-azido-FMN is the OH group of Tyr-375. The oxygen atom from this group is positioned above the si face of the flavin, near C6. Although this atom is 3.8 Å away, Tyr-375 is solvent-exposed and is not constrained by other interactions and thus may have some freedom to move and react at C6. The only other atoms within a reasonable distance are a carbonyl oxygen, a main chain nitrogen, and aliphatic and aromatic carbon atoms, none of which would be reactive. In the absence of a crystal structure of OYE containing the modified flavin or a direct chemical identification of the reactive side chain, the identity of the residue

**FIG. 2.** Resonance structures possible for the oxidized form of the flavin isoalloxazine ring.

**FIG. 3.** Resonance structures for (a) 8-azido-FMN and (b) 6-sulfoxo-FMN.
cannot be resolved. It is possible that the Tyr-375 OH is also involved in stabilizing the sulfenate species by donating a hydrogen bond to the O⁻. In addition, the protein’s stabilization of resonance forms D and H (Fig. 2) places a partial positive charge on C6 that would also stabilize the sulfenate and destabilize the sulfoxide (Fig. 3b). From this analysis, it is clear that it is not necessary to have a positively charged amino group nearby to explain the results of the modified flavin studies.

N5—At N5, the NMR work provided relatively strong evidence that there was a hydrogen bond from the protein to N5, but the idea that the shift was caused by a positive charge nearby could not be ruled out (11). The hydrogen bond interpretation was later discounted because the pKₐ of N5 in oxidized flavin was −3.8 (22), and it was thought that this made a hydrogen bond to N5 unlikely (1). Also, because a positive charge was suggested to be present near C6 (2), it would also be near N5 and could explain the NMR data. In the crystal structure, there are no positively charged residues nearby, but there is a strong hydrogen bond from the main chain nitrogen of Thr-37 to N5. Although there was no precedent for such a hydrogen bond to N5 at the time, since then it has become clear that such hydrogen bonds occur in several flavoproteins, among them ferredoxin reductase (23) and flavocytochrome b2 (24), so the argument that such an interaction cannot form is not justified. (In this respect it may be noted that carbonyl oxygens, which have pKₐ values in the range from −2 to −7, are known to be good hydrogen bond acceptors.) Thus, it is clear that the NMR, modified flavin, and crystallographic results are in agreement, but that the lack of an appropriate precedent led the interpretation astray.

C4, O4A—Both NMR and modified flavin studies are supported by the crystal structure, showing hydrogen bonds from the protein to O4A (from OY of Thr-37 and the main chain nitrogen of Gly-72). However, contrary to previous postulations, there is no thiol nearby. When 4-thio-FMN is bound to OYE, it slowly reverts back to FMN, and this was thought to indicate the presence of a protein thiol (4). In the crystal structure, there are no thiols near the flavin (or even in the whole protein); the only nearby nucleophile is the hydroxyl of Thr-37 (3.8 Å), or a water molecule that could attack from the accessible si side of the flavin. Although less favorable than a reaction with a thiol, it is possible for secondary alcohols to be involved in such hydrolisis reactions. Whereas it is possible that a thiol exists in the active site of the other OYE isozyme from S. carlsbergensis, as noted above, the uniform behavior of OYE in this experiment and the high conservation of OYE active site residues argue against this explanation.

Conclusions—It is clear that the modified flavin and NMR studies provided a wealth of information about the flavin binding site in OYE. The majority of the information determined by these methods was confirmed by crystallography, underscoring that these are reliable and valuable approaches for studying the flavin environment. Especially useful were the determinations of solvent accessibility, proximity of the flavin to protein atoms, and hydrogen bonding. However, care must be taken when interpreting the data from spectroscopic studies to note where ambiguities in interpretation exist and to take into account the stabilization of flavin resonance forms by protein groups. The changes in the charge distribution due to the stabilization of a class of resonance forms leads to changes in the reactivity and thus the apparent environment of particular atoms.

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