First publ. in: Biochemical Journal 239 (1986), 1, pp. 1-12

New flavins for old: artificial flavins as active site probes of flavoproteins

Sandro GHISLA and Vincent MASSEY*

Fakultät für Biologie, Universität Konstanz, D-7747 Konstanz, Federal Republic of Germany, and Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, U.S.A.

INTRODUCTION

Flavin enzymes are unique as biological catalysts in that they carry out a wide variety of different biochemical processes. This sets them apart from enzymes which use other coenzymes, like pyridine nucleotides, pyridoxal phosphate or thiamin pyrophosphate, each of which involves a single type of chemical event. Flavins thus are involved in the dehydrogenation of a variety of substrates, in one-electron transport processes, in the activation of molecular oxygen, in the emission of biological light (bioluminescence), in photobiochemical processes (e.g. phototropism), and probably in processes involving control of enzymic functions. For a discussion of this topic and of the classification of flavin enzymes we refer to some recent reviews [1–4].

Modes of interaction of flavin with protein

The selection of which of this variety of functions is carried out by a particular enzyme is clearly brought about by the interaction of the isoalloxazine nucleus of the flavin with the protein, and presumably requires very fine tuning. The isoalloxazine moiety of the flavin, i.e. the part of the molecule which is involved in catalysis, offers several possibilities for interaction with various protein functions. It is, chemically speaking, amphipathic, i.e. the xylene moiety is hydrophobic and prone to interact with hydrophobic protein areas, whereas the pyrimidine ring is relatively electron-deficient and hydrophilic, and is comparable with pyrimidine bases in its capability to form hydrogen bridges (Fig. 1). The flavin can exist in three different redox states, the oxidized, semiquinoid, and fully reduced forms. At each of these levels the chemical properties are very different, particularly those of the pyrimidine moiety. This part of the molecule becomes electron rich in the reduced state and has a negative charge ($pK \sim 6.5$) at neutral pH [5]. Also, the flavin N(5) is not particularly basic in the oxidized state and thus not prone to undergo interactions with the protein. In contrast to this, N(5)–H of the flavin semiquinone has a pK_a of ~ 8–8.5 [6,7], the pK_a of the same function in the fully reduced form is probably > 16, and the pK_b ~ 3 [8]. From this it is apparent that substantially different modes of hydrogen bond interaction with the protein can occur with the different redox

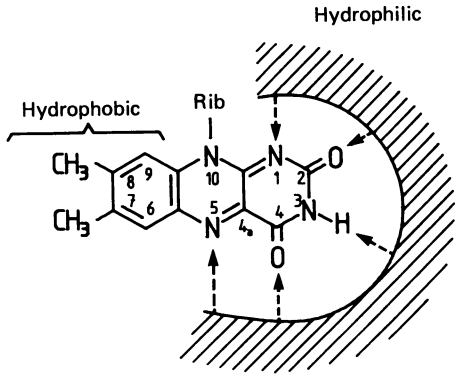


Fig. 1. Representation of possible flavin–protein interactions

The hatched portion is meant to indicate that interactions with this region will be largely hydrophilic, and the open region to indicate mainly hydrophobic interactions. In neither case is anything inferred about solvent accessibility to the various parts of the flavin structure.

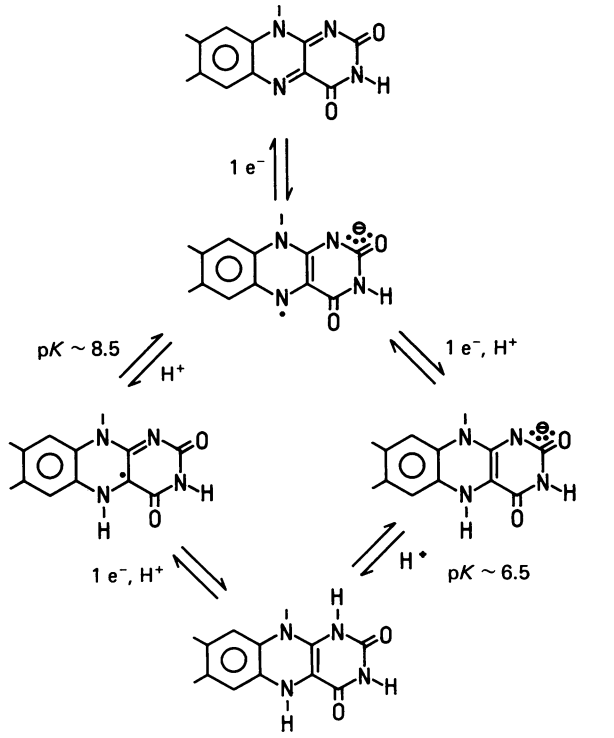


Fig. 2. Redox and ionization states of flavins

The forms shown are those likely to play a role in reactions catalysed by flavoproteins.

* To whom correspondence and reprint requests should be addressed in Ann Arbor.

states of the flavin. These possibilities can be deduced from Figs. 1 and 2.

In order to understand the mechanisms which govern flavin reactivity, knowledge of the protein environment at the active centres of different classes of enzymes is necessary. The ultimate method for studying enzyme active centres, and the interaction of protein, substrate and cofactors is, of course, X-ray crystallography. This technique, however, involves either very considerable effort in time or amount of protein required for the measurements, and in many cases cannot be used because of lack of suitable crystals.

Modes of studying the active centre of flavin enzymes

A valuable supplement to crystallography, which requires much less time and only small amounts of protein, is the replacement of the native flavin by appropriately modified flavins (see [1,2,9] for reviews). This is facilitated by the ease by which the cofactor can be reversibly removed from a large number of enzymes, in which it is not covalently bound [10,11].

The first enzyme studies involving the use of chemically modified flavin coenzymes date from the mid 1960's and were aimed at elucidating the factors required for tight binding to the apoprotein. They involved variations in the chain length, and of the constitution of the N(10) side chain [12], modifications of the adenine moiety [13], or of positions 2 and 3 of the isoalloxazine [14]. Later, the modes of binding of flavin to flavodoxins were studied by using flavins modified at position 6 and 4, i.e. iso-FMN and 3,4-dihydro-FMN [15]. The first report dealing with the role of a specific flavin function was that by Edmondson *et al.*, which addressed the role of N(5) and involved the use of 5-deaza-FMN and a flavodoxin [16]. It was followed by a long series of papers dealing with the same 5-deazaflavin model and its use for the study of biochemical mechanisms (see [1] and [17] for reviews on 5-deazaflavins). Studies on the interactions of flavin with the protein active centre by using spectral probes were initiated by the use of the naturally occurring 6-OH and 8-OH-flavin chromophores [18,19].

Types of modified flavins suitable for the study of enzyme active centres

A variety of problems can be addressed conveniently by using modified flavin coenzymes as probes. The latter can be classified in four broad categories on the basis of the type of information which is obtained by their use.

Spectral probes. These analogues yield information by virtue of spectral effects induced upon binding of the coenzyme and on the effects caused by addition of substrates or inhibitors, changes in pH, etc.

Chemically active probes. In these cases a functional group of the flavin has been modified in such a way as to be able to react either with the protein or with solvent-borne reagents. Such a probe can yield information by covalently reacting with amino acid residues (labelling of the active centre), or can give information on the accessibility of a particular flavin position to the solvent.

'Mechanistic probes'. This class can include members of any of the other classes as well. Its main characteristic is a modification of the mode or rate of reaction of the coenzyme with substrate induced by the chemical modification of the flavin. Typical cases include flavins in which the redox potential has been altered, or 5-deazaflavins in which the course of (covalent) catalysis at position 5 can be altered.

Photoaffinity labels. Members of this category will carry a functional group which can be photoactivated, and which subsequently might react with either a protein residue, or with solvent molecules.

Strategies for the preparation of modified flavins

Two problems are encountered in the preparation of modified flavin coenzymes suitable for enzymic work: the isoalloxazine moiety must be modified chemically, and the riboflavin analogue must be converted into the FMN or FAD derivative. The preparation of analogues having a substituted ring atom, or involving the substitution of one of the methyl groups at positions 7 or 8, invariably involves full synthesis. Substitution of the oxygens at positions 2 or 4, as well as the introduction or exchange of substituents at positions 6, 8, or 9, can be accomplished with the preformed isoalloxazine molecule. For further details see [4]. The phosphorylation of the riboflavin 5′ position to yield FMN, and the subsequent coupling with AMP to form the FAD derivative, has long been the major obstacle for the use of modified flavins in enzymology. The chemical synthesis has proven cumbersome, and too harsh for the preparation of most analogues, and is of little practical use. The first riboflavin kinases and FAD synthetases to be used were also of rather limited use due to their narrow specificity for riboflavin analogues and difficult preparation [11]. A breakthrough in this field was the description by Walsh and colleagues of an easily obtainable FAD synthetase from *Brevibacterium ammoniagenes*, which exhibits very broad specificity, and is comparatively stable [20]. This enzyme has been purified recently to homogeneity (D. J. Manstein & E. F. Pai, unpublished work). In some cases, in which the enzyme would not accept riboflavin derivatives as substrates, the preparation of FAD analogues modified at position C(6) has been achieved by chemical conversions at the FAD level [21].

SPECTRAL PROBES

The concept behind this approach requires a flavin chromophore which, at the active centre, will be sensitive to the properties of the protein environment, without chemically reacting with it. The polarity of the active centre (hydrophobic/hydrophilic) is often reflected by the 'resolution' of the flavin spectrum in the visible range. Lipophilic flavins in solvents like cyclohexane or toluene have two well resolved bands at 472 and 447 nm, while the same chromophore in aqueous solvent has a $\lambda_{max.}$ at 445 nm [22]. The spectrum of some flavin enzymes like yeast glutathione reductase or of some flavodoxins is of the resolved type, suggesting a non polar environment of the flavin [23]. With other enzymes like D-amino acid oxidase [24] or lactate oxidase [25] this effect is induced by binding of inhibitors. Some modified flavin coenzymes, such as flavin 2-sulphoxides [26] also exhibit strong solvatochromy, and can be used to probe the environment polarity at specific positions.

Information on the presence of dipoles, of charges, or of hydrogen bonds, which interact with the flavin at the

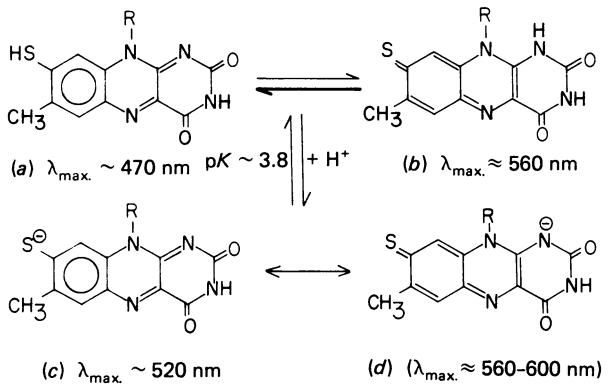


Fig. 3. Tautomeric and mesomeric forms of 8-mercaptoflavin

(From [28]).

enzyme active centre, can be obtained by using chromophores which exist in different tautomeric and/or mesomeric forms. In this case the protein is expected to affect the equilibria between these forms. For the interpretation of results the spectra of the different chromophores must be known from model studies. This has been done for 8-mercaptoflavin, a molecule which can be described by the extreme forms shown in Fig. 3 [27,28]. The special features of this molecule are the large spectral difference between the neutral (*a*) and the anionic (*c,d*) forms ($pK_a \sim 3.8$), as well as between the corresponding 'benzenoid' (*a,c*) and 'paraquinoid' (*b,d*) forms. While the absorption spectra of the tautomeric forms (*a*) and (*b*) were obtained relatively easily from the alkylated derivatives, the spectral differentiation between the mesomeric forms (*c*) and (*d*) was more difficult. The attributions shown were confirmed by binding to flavoenzymes where the topography of the active centre was known from X-ray crystallography. Thus, with *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*, and with glutathione reductase from human erythrocytes, in which dipoles formed from a protein α-helix exert a partial positive charge directed toward the flavin N(1)–C(2)=O position [29–31], the spectrum

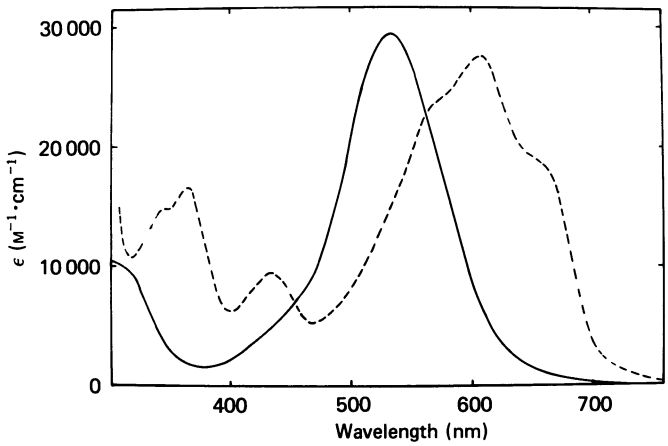


Fig. 4. Spectral changes accompanying the binding of 8-mercapto-FAD to the apoprotein of glucose oxidase

Solid curve, 8-mercapto-FAD; broken curve, bound to apo-glucose oxidase. (From [28]).

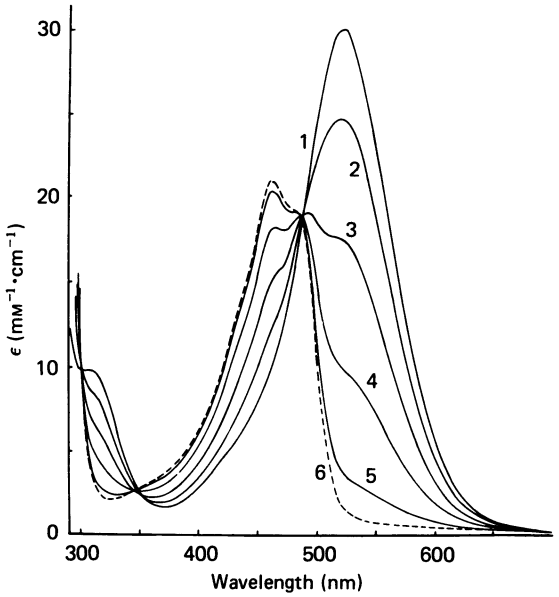
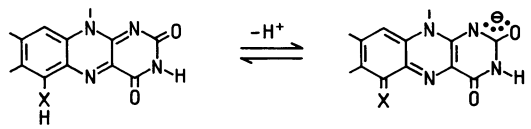


Fig. 5. Titration of 8-mercaptoriboflavin with egg white riboflavin-binding protein

Curve 1, 8-mercaptoriboflavin; curves 2–6, after successive additions of 0.24, 0.48, 0.71, 095 and 1.19 mol of protein/mol of 8-mercaptoriboflavin. (From [28]).

predicted for (*d*) was obtained [28,32], and with flavodoxin, in which the flavin 8-position is exposed to solvent [33], the spectrum predicted for (*c*) was obtained [28].

The dramatic effects occurring upon binding 8-mercaptoflavins to proteins are exemplified by glucose oxidase and riboflavin-binding protein. With glucose oxidase, as with many other oxidases [28], the paraquinoid resonance contributor (*d*) appears to be stabilized (Fig. 4). This should be contrasted with the effects observed upon binding of 8-mercaptoriboflavin to riboflavin-binding protein (Fig. 5), where the spectrum typical for form (*a*) is obtained, in agreement with the known preference of this protein for binding neutral flavins [21,34]. From these considerations it is apparent that 8-mercaptoflavins can yield very important information on the localization of charge dipoles/hydrogen bridges at the active centre of flavin enzymes. The same type of information can be obtained with flavins which have been modified following the same concept, but using other functions, and at different positions. 8-Hydroxyflavins could theoretically be described by similar canonical structures and mesomeric forms as described in Fig. 3 for 8-mercaptoflavin. In this case, however, the spectra of the ionized species of the free flavin [19] are consistent with the charge being located predominantly in the pyrimidine ring, i.e. with a paraquinoid structure.



X = O, pK = 7.1; X = S, pK = 5.9

Fig. 6. Structures of the neutral and predominant mesomeric anionic forms of 6-hydroxy- and 6-mercaptoflavins

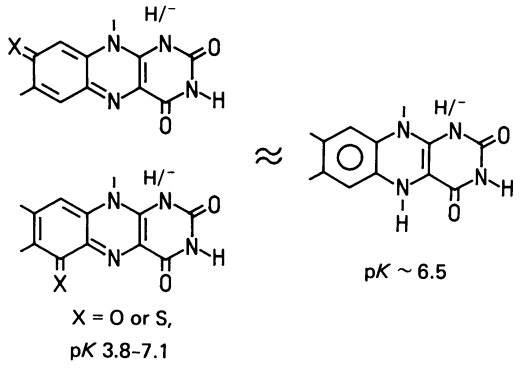


Fig. 7. 6- and 8-oxygen and sulphur-substituted oxidized flavins as analogues of reduced normal flavins

This might be ascribed to the differences of sulphur and oxygen in undergoing π-interactions and in electronegativity. 6-Hydroxy- [18] and 6-mercaptoflavins [21] (Fig. 6) can be described by a similar set of structures as the 8-substituted analogues (cf. Fig. 3). The neutral molecules are protonated at position 6α in free solution, while the anions are characterized by longwavelength absorption, which is typical of N(1)-blocked 6-hydroxyflavins [35]. This suggests that in 6-substituted flavins the paraquinoid form shown in Fig. 6 yields a major contribution to the charge distribution (without requiring stabilization by protein interactions).

Further interesting information can be obtained on the microenvironment by determining the shifts in pK of these oxidized flavin analogues upon binding to apoproteins. The derivatives mentioned above have pK values of 3.8 (8-SH), 4.8 (8-OH), 5.9 (6-SH), and 7.1 (6-OH), covering an appropriate range near neutrality. It should be pointed out, that with these ionizable flavins, it is the ionization process of normal reduced flavin that will be mimicked (Fig. 7). The independent determination of the pK of unmodified reduced flavin is much more difficult than that of the analogues due to the high oxygen sensitivity of the former.

Thus any flavin–protein interaction which stabilizes the reduced flavin anion, such as a protein positively

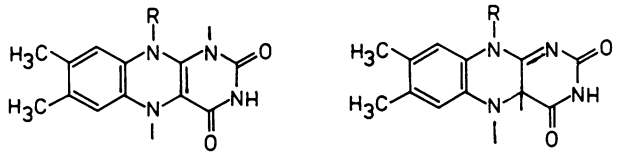


Fig. 8. Structures of 1,5-dihydro- (left) and 4a,5-dihydroflavin chromophore (right)

charged residue in the vicinity of the flavin N(1) position, should also lower the pK of these 6- and 8-substituted flavins, and stabilize the benzoquinoid anion form of 8-mercaptoflavin [Fig. 3, structure (*d*)]. Such an interaction has been suggested to facilitate the uptake of redox equivalents through position N(5) during catalysis [3,4,36], and should thus parallel the redox potential of the flavin enzyme. Such a correlation has been documented in several cases [28]. Conversely, with *Megasphaera elsdenii* flavodoxin, in which the redox potential of the flavin is lowered [37], the pK of all four analogues bound to the protein is increased, although to a different degree (see Table 1). It thus appears that indeed the shift of the microscopic pK values of the analogues at the enzyme active centre will reflect that of normal reduced flavin bound to the same enzyme. A direct spectroscopic probe for the determination of the ionization state of the reduced flavin is 4-thioflavin, for which the spectral differences between the anionic and neutral forms are much more pronounced than with normal flavin [38].

The differentiation between 1,5- and 4,6-dihydroflavin chromophores (Fig. 8) by using spectroscopic methods has proven rather difficult with normal flavins [22]. This differentiation is much easier with 2-thioflavins, since the spectra of the two tautomers [as exemplified by stable adducts at the N(5)- and C(4a)- positions] are distinctly different in this case [39].

CHEMICALLY REACTIVE PROBES

Although 8-halogen-substituted flavins had long been available and employed in nutritional and metabolic studies [40], their chemical reactivity was not recognized until 8-chloro-FAD was incorporated into lipoyl dehydro-

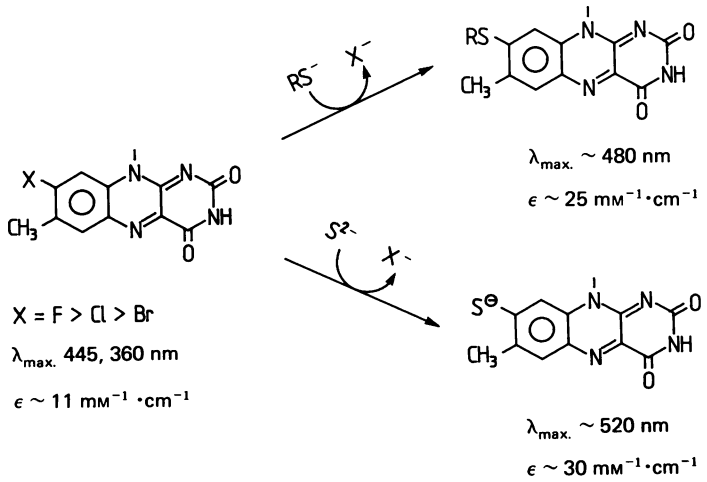


Fig. 9. Nucelophilic displacement reactions of 8-halogen-substituted flavins

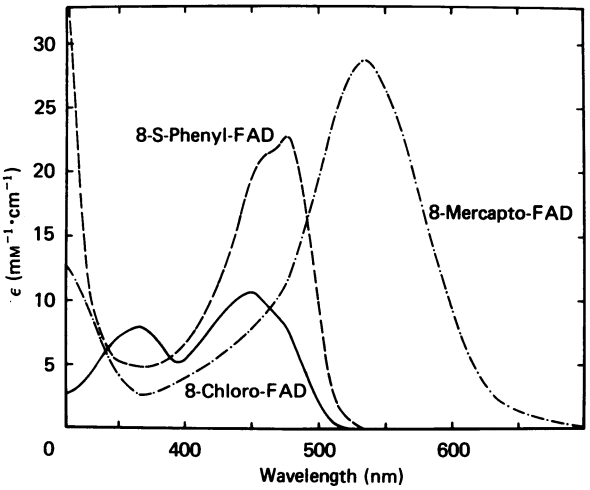


Fig. 10. Spectra of 8-chloro-FAD and the reaction products with thiophenol and Na_2S

(From [43]).

genase and large spectral changes were found to take place, due to covalent fixation of the flavin to a protein thiolate residue, with the elimination of Cl^- [41]. This provided a facile method for the production *in situ* of a large number of 8-S-substituted flavins, including 8-mercaptoflavins (see previous section), with very large changes in absorption spectrum [27,28], (Figs. 9 and 10). Thus 8-halogen-substituted flavins can be used as active site probes of flavoproteins in two different ways. If the active site has a suitably positioned thiolate residue, it would be expected to displace halide from 8-halogeno-flavin, and give a covalently bound 8-S-flavin. Of the many cases in which 8-chloro- or 8-fluoroflavins have been introduced into apoproteins, only with lipoyl dehydrogenase [41] and the electron transfer protein [9,42] has such a covalent binding been found. In the remaining cases the 8-halogen-substituted flavin is tightly bound and gives only the minor spectral changes normally associated with binding of flavins to their apoproteins. In these cases the accessibility of the flavin 8-position to solvent-borne reagents can be tested by comparison of the rates of reaction of the protein-bound and free 8-substituted flavin with thiols such as thiophenol, or with Na_2S [27,28,43]. The nucleophilic displacement of halide by thiolates presumably proceeds through a tetrahedral intermediate; hence failure to react with a protein-bound 8-halogen-substituted flavin may imply that the protein structure will not accommodate the tetrahedral intermediate, rather than giving definitive evidence that the flavin 8-position is inaccessible. Fortunately this possibility is easily tested via the chemical reactivity of 8-mercaptoflavins, where reaction with alkylating agents such as iodoacetic acid or iodoacetamide does not involve flavin tetrahedral intermediates. In this case the influence of charged protein residues as well as the charge localization in the flavin itself would also be expected to show up in differential reactivities with charged versus uncharged alkylating agents.

The wealth of information coming from studies of 8-mercaptoflavins bound to specific apoproteins encouraged us to use the same principles with 2-thioflavins and 4-thioflavins. With 2-thioflavins the reaction rates with iodoacetic acid and iodoacetamide are too low to make these convenient reagents for probing solvent accessibility. However, methylmethane thiolsulphonate (MMTS) does react rapidly, and serves as a suitable probe [44]. It was also found that 2-thioflavins react readily with H_2O_2 and peracids such as *m*-chloroperbenzoate to form a remarkably stable S-oxide intermediate on the way to higher oxidation states. The latter are unstable and hydrolyse to yield the normal (2-oxo) flavin coenzyme [26]. The flavin 2-S-oxide has a high absorption coefficient ($\sim 10000\ M^{-1}\cdot cm^{-1}$) with a $\lambda_{max.}$ in the region 550–650 nm, the position of the $\lambda_{max.}$ depending on whether the solvent is polar or non-polar. Thus the reaction of enzyme-bound 2-thioflavins with peroxides or peracids gives information about solvent accessibility, and the spectral properties of enzyme-bound flavin 2-S-oxides give information about whether the protein environment is hydrophobic or hydrophilic.

4-Thioflavins show similar properties to those of 2-thioflavins, but with enhanced reactivity toward nucleophiles. In addition to the use of methylmethane thiosulphonate, H_2O_2 and *m*-chloroperbenzoate as solvent-borne probes, the reactivity with primary and secondary amines, hydroxylamine, and thiols is sufficiently great to make them useful reagents [38]. Sulphite also reacts readily to give either N(5)-sulphite adducts of the 4-thioflavin, or the 4-hydroxy-4-sulphonyl flavin, depending on the protein, while in the free system also the flavin 4-thiosulphate is formed as an intermediate [45]. The reaction of 4-thioflavins with amines results in quite stable 4-aminoflavins. Thiols, on the other hand, catalyse the conversion of 4-thioflavins to normal (4-oxo) flavin. Thus an apoprotein with a suitably positioned thiol residue in the active site will bind the appropriate 4-thio-FMN or 4-thio-FAD and then convert it to the normal flavoenzyme at a rate which is presumably determined by the relative positions of the protein thiol and the sulphur atom of the 4-thioflavin. Of the proteins so far studied, 4-thio-FMN lactate oxidase is converted slowly to the normal FMN form in charge transfer complex with a newly generated protein persulphide, and 4-thio-FAD glucose oxidase is converted very rapidly to the normal enzyme [35].

Accessibility to the flavin N(5)-position in native flavoproteins is clear in those cases which form N(5)-sulphite adducts [46]. This is the case with many flavoprotein oxidases, and there is considerable evidence that this is due to the effect of a positively charged protein residue, which has an inductive effect on the nucleophilic attack of sulphite at the flavin N(5)-position, and which stabilizes the resultant anion with its negative charge in the N(1)–C(2)=O locus as shown in Fig. 11 [28,36,46,47].

While the enzymes which form N(5)-sulphite adducts obviously must have solvent accessibility to the flavin N(5) position, lack of sulphite adduct formation does not necessarily mean that the position is inaccessible. Fortunately another accessibility probe for this position is made available by the reaction of peroxides and peracids with 5-deazaflavins [48]. This results in the formation of a 4a,5-epoxide derivative, absorbing maximally around 340 nm. In some enzymes and in model compounds at high pH, this is followed by a ring-opening reaction involving the pyrimidine ring, with subsequent decarboxylation, elimination of the N(3)-residue, and ring closure to give a condensed structure

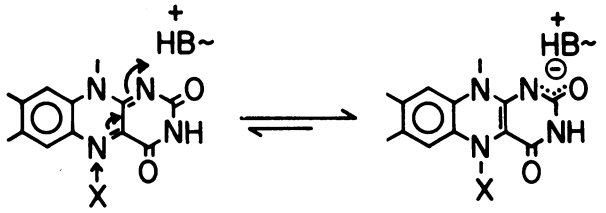


Fig. 11. Mode of reaction of sulphite and other nucleophiles with oxidized flavin

The positive charge close to the pyrimidine nucleus is shown to stabilize the negative charge in the adduct, and might thus facilitate its formation.

with intense fluorescence and a very narrow, complex absorption spectrum [49].

Recently we have also begun to study enzymes reconstituted with 6-thiocyanatoflavins and 6-mercaptoflavins (V. Massey, S. Ghisla & K. Yagi, unpublished work) [21]. These flavins and some of their properties had previously been reported in connection with the structural determination of the covalently bound flavin of trimethylamine dehydrogenase [50,51]. They promise to be very useful for probing the active site environment around the flavin 6-position, a position of interest because of its close proximity to the redox-active 5-position. 6-Thiocyanatoflavins have been found to be converted rapidly to 6-mercaptoflavins by reaction with thiols such as dithiothreitol, with large and distinctive changes in absorption spectrum (Fig. 12). This provides a convenient probe for solvent accessibility to the flavin 6-position in specific flavoproteins. When 6-SCN-FAD is bound to apo-D-amino acid oxidase there is a slow, spontaneous conversion of the bound flavin to 6-mercapto-FAD, indicating the presence in the active site of a protein cysteine residue. A similar phenomenon is found with 6-SCN-FMN bound to lactate oxidase, where the conversion is much more rapid, implying that the active site cysteine residue is located close to the flavin 6-position. The reaction of 6-SCN-flavins with dithiothreitol makes possible the easy preparation of 6-mercaptoflavins for incorporation into apoproteins, since when excess thiol is used and trace metal catalysis of thiol oxidation is slowed by the addition of EDTA, the 6-mercaptoflavin is stabilized against oxidation to the disulphide dimer, and any dimer which is formed is rapidly re-reduced by the excess dithiothreitol. 6-Mercaptoflavins react readily with iodoacetamide (see Fig. 12), and very rapidly with methylmethane thiolsulphonate and *N*-ethylmaleimide [50]. They also react readily with H_2O_2 and peracids, yielding spectroscopically distinctive products at the 2 e^- oxidation level (sulphinate and sulphonate). Thus there exists a wide variety of reactions with 6-mercaptoflavin and reagents of different size and charge which should provide detailed information about the accessibility of different types of molecules at the flavin 6-position.

MECHANISM PROBES

In principle, any flavin analogue with features chemically distinctive from those of the normal coenzyme can be used in the study of reaction mechanism, provided the function in question is involved in the catalytic process. One of the most widely studied flavin analogues and historically the first used to probe specific mechanistic questions is 5-deazaflavin (cf. Fig. 13). The principal use of this flavin has involved three basic problems. The first pertains to the question whether covalent intermediates occur during catalytic dehydrogenation reactions (carbanion mechanism) or whether hydride transfer takes place [1,20]. If a covalent adduct was to be formed as shown in Fig. 13, then the intermediate resulting with 5-deazaflavin would not be prone to fragmentation [breaking of C(5)–C bond] to yield the products.

It is expected that an intermediate such as that shown would be stable, and accessible to isolation and structure identification. However, most enzymes reconstituted

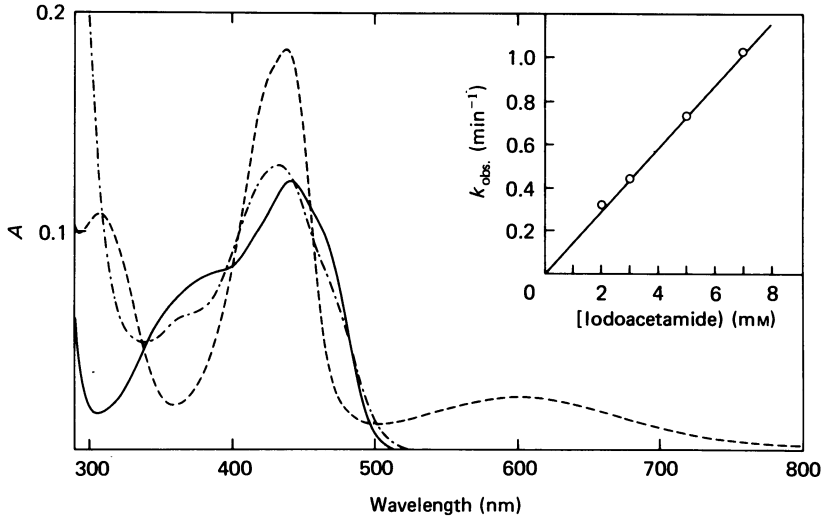


Fig. 12. Spectra of 6-SCN-riboflavin and derivatives

Solid curve, 6-SCN-riboflavin; broken curve, 6-mercaptoriboflavin; broken/dot curve, 6-SCH_2CONH_2-riboflavin. The inset shows the second order kinetics of reaction of iodoacetamide with 6-mercaptoriboflavin. (From [21]).

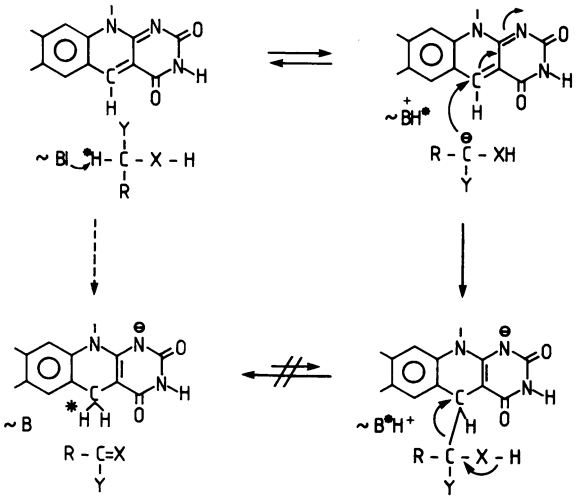


Fig. 13. Expected reactions of 5-deazaflavin substituted-enzymes

Substrates that are oxidized via a carbanion pathway are expected to react as shown on the right, those by a hydride-transfer mechanism as on the left.

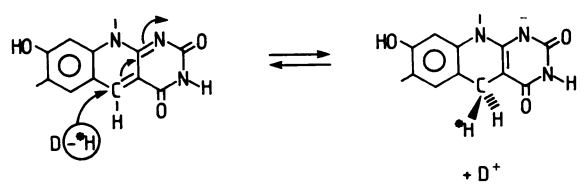


Fig. 14. Stereochemistry of hydrogen transfer from substrate to protein-bound 8-hydroxy-5-deazaflavin

The stereospecificity shown is that of transfer to the *si*-face of the flavin.

with oxidized 5-deazaflavin react only very sluggishly with substrates which are potentially able to generate stabilized carbanions. The reverse reaction, i.e. the reoxidation of reduced 5-deazaflavin by products, proceeds much faster. This is, at least in part, due to the lower redox potential of the 5-deazaflavin couple compared with that of normal flavin [52]. Importantly, however, in no case has a covalent adduct ever been observed. Furthermore the substrate α-proton was incorporated into the 5-deazaflavin position C(5) in both D-amino acid oxidase [53,54], and lactate oxidase [55], enzymes which are commonly believed to involve a carbanion mechanism. As discussed by several authors [3,56,57] these results have posed a mechanistic dilemma which has not yet been resolved. They would appear to exclude a carbanion-initiated dehydrogenation mechanism, which *a priori* is not expected to lead to incorporation of the α-hydrogen of the substrate into the flavin. Such a mechanism is, however, the one which best explains a multitude of other results, and which is accepted by general consensus for the dehydrogenation of substrates carrying an activated (acidic) proton [3]. It has been pointed out frequently that the 5-deazaflavin system can also be considered to be an analogue of nicotinamides as well as flavins [1,17]. This analogy, which has several arguments in its favour, would however have far-reaching consequences, if it is taken as implying that upon substitution of normal flavin with 5-deazaflavin the mechanism of catalysis is switched to that of pyridine nucleotides, i.e. hydride transfer. It would imply that at the same enzyme active centre a catalytic machinery exists which would catalyse oxidation processes via opposite mechanisms (carbanion versus hydride transfer), a rather unlikely possibility.

With (potential) hydride donors such as pyridine nucleotides, the 5-deazaflavin will accept a hydride at position C(5), and in the reduced 5-deazaflavin formed, the two C(5) hydrogens should be non-equivalent with respect to the face of the flavin on which they have been transferred (Fig 14). This concept was originally put forward and discussed by Walsh, but was thwarted experimentally by the rapid scrambling of hydrogen in mixtures of oxidized and reduced 5-deazaflavin [20]. The problem has recently been resolved using 8-hydroxy-5-deazaflavin (D. J. Manstein, E. F. Pai, L. M. Schopfer & V. Massey, unpublished work). This was made feasible experimentally by the finding that 5-deaza-FAD general acyl-CoA dehydrogenase was reduced $> 90\%$ specifically on one face with borohydride [58]. In the similar reaction with 8-hydroxy-5-deazaflavin, it was found that this face was the same *re*-face as that reacting with NADPH in glutathione reductase where the stereospecificity was known from the X-ray crystallographic structure [30].

The third mechanistic application of 5-deazaflavins is based on a major chemical difference between normal flavin and the 5-deaza analogue, the ability to stabilize radicals and to carry out 1 e^- transfers. Indeed, this is one of the most important properties of normal flavin.

$$Fl_{ox.} + 1\,e^- + H^+ \leftrightarrow FlH^{\cdot} + 1\,e^- + 1\,H^+ \leftrightarrow FlH_2$$

This property is completely missing in 5-deazaflavins, since semiquinone formation with 5-deazaflavins is energetically highly unfavourable, thus precluding any role of 5-deazaflavin radicals in 1 e^- catalysis [52]. Thermodynamically the reaction of reduced 5-deazaflavin with oxygen is even more favourable than that with normal reduced flavin ($E_0' = -300$ mV versus -210 mV). The incapability of this analogue to undergo 1 e^- processes will preclude spin inversion catalysis by exchange of electrons during the activation of O_2 (cf. the concluding section). This is the likely reason for the lack of reaction of reduced 5-deazaflavin with O_2. 5-Deazaflavin is thus a good probe for the differentiation between 1 e^- and 2 e^- processes. It has been used for example to demonstrate that during α,β-dehydrogenation of acyl-CoA substrates the β-hydrogen of substrate is transferred directly to the flavin position 5 as a hydride [58].

The stability of reduced 5-deazaflavin towards O_2 also makes it a convenient substitute for normal fully reduced flavin. For example, this has made it possible to determine that the function of normal flavin in the unusual enzyme glyoxylate carboligase does not involve a redox reaction, since replacement of the normal flavin with oxidized or reduced 5-deazaflavin results in retention of 50–100% catalytic activity [59]. It should be emphasized that with all other enzymes substituted with 5-deazaflavins, only partial reactions have been observed [17].

In contrast, 1-deazaflavins retain most of the chemical reactivities of normal flavins, including rapid reaction of the reduced flavin with O_2, and thermodynamic

stabilization of the semiquinone form [60]. 1-Deaza-FAD-reconstituted glucose oxidase has a catalytic activity ~10% that of the native enzyme, with the lower activity presumably due to the lower redox potential (E'_0 of −280 mV compared to −210 mV for FAD). However, 1-deaza-FAD D-amino acid oxidase has the same catalytic activity with D-alanine as native enzyme [61], in agreement with previous conclusions from kinetic studies of the native enzyme that the rate-limiting step in catalysis is the physical release of product [62,63]. The same conclusion was reached from studies on the enzyme substituted with 7,8-dichloro-FAD [64].

Particularly interesting results have come from the use of 1-deazaflavin with flavoprotein mono-oxygenases. The 1-deazaflavin form of cyclohexanone mono-oxygenase is fully competent in carrying out the normal mono-oxygenation reaction of this enzyme [65], and reduced 1-deaza-FMN is competent in the bioluminescence reaction of bacterial luciferase [66]. In contrast, when 1-deazaflavin is introduced into all aromatic hydroxylases so far studied, i.e. orcinol hydroxylase [61], *p*-hydroxybenzoate hydroxylase [67], melilotate hydroxylase [68] and phenol hydroxylase [68], the substrate-controlled oxidation of NAD(P)H characteristic of the native enzymes is observed, but no hydroxylation. With *p*-hydroxybenzoate hydroxylase, the reduced enzyme–substrate complex reacts rapidly with O_2 to form a 1-deazaflavin-C(4a)-hydroperoxide, in an analogous fashion to that with native enzyme. However, with the 1-deazaflavin enzyme the hydroperoxide is not capable of carrying out hydroxylation of the substrate; instead it breaks down in a non-productive fashion to regenerate oxidized 1-deazaflavin and H_2O_2 [67]. While the explanation for these results is not yet clear, it is obvious that different pathways in the oxygen activation reaction are followed in the presumed electrophilic oxygen insertion reactions catalysed by the aromatic hydroxylases, and the nucleophilic attack of the flavin hydroperoxide on substrate which presumably occurs in the cyclohexanone mono-oxygenase and luciferase reactions.

1-Deazaflavin has also been used successfully to determine whether the bioluminescent process of bacterial luciferase proceeds by rearrangement and ring opening mechanisms involving the pyrimidine or the pyrazine moieties of the flavin [66]. The fact that 1-deazaflavin is a competent light emitter in the luciferase reaction and that the spectrum of the emitted light is similar to the luminescence emission spectrum of 4a,5-dihydro-1-deazaflavin demonstrated clearly that many proposed mechanisms for this system were incorrect [66].

Isoflavins (6-methyl-8-nor-flavins) have been used successfully, in isolated cases, to determine whether position C(6) of the flavin is involved in specific enzymic processes. For example, D-lactate dehydrogenase is irreversibly inhibited by α-hydroxybutynoate to yield a pink chromophore [69]. The determination of its structure was difficult, in view of the small quantities of material available. By substituting normal FAD with iso-FAD it could be shown that the pink chromophore was not formed, indicating a direct involvement of position C(6) in its genesis. The structure of the inactivation product was subsequently shown by n.m.r. to involve formation of an aromatic ring between C(6) and N(5) as shown in Fig. 15 [70].

The availability of flavin analogues with different redox potentials has also permitted important specific questions to be addressed. Depending on the nature and position of the substituent, ring-modified flavins are available with redox potentials ranging from approx. −370 mV (1,5-dideazaflavin) to −30 mV (8-phenylsulphonylflavin) (for useful tables of redox potentials see [27,71–73]). Such an approach has been used to obtain convincing physical evidence that the long wavelength bands formed on adding phenols to Old Yellow Enzyme are due to charge transfer transitions involving the phenolate anion as donor and oxidized flavin as acceptor [72,74]. A similar series of flavins at the FMN level was used as a replacement of the native FMN in NADPH–cytochrome *P*-450 reductase. The results support strongly the concept that the FMN and FAD components of this enzyme have separate roles in catalysis, with FAD being the flavin reacting with NADPH and $FMNH_2$ the flavin reacting with cytochrome *P*-450 [75]. Very recent studies with a bacterial electron-transferring flavoprotein, in which the native FAD was replaced by 8-chloro- or 8-fluoro-FAD, resulted in differential covalent attachment of the flavin, depending on conditions, with strong indications that the two flavins of the dimeric protein have different catalytic functions [42].

Lack of space prohibits details of other examples where comparisons among a series of flavin analogues have produced information not readily available by other means. Examples are NADPH–adrenodoxin reductase [73], xanthine oxidase [76], ferredoxin–NADP reductase [77] and general acyl-CoA dehydrogenase [78].

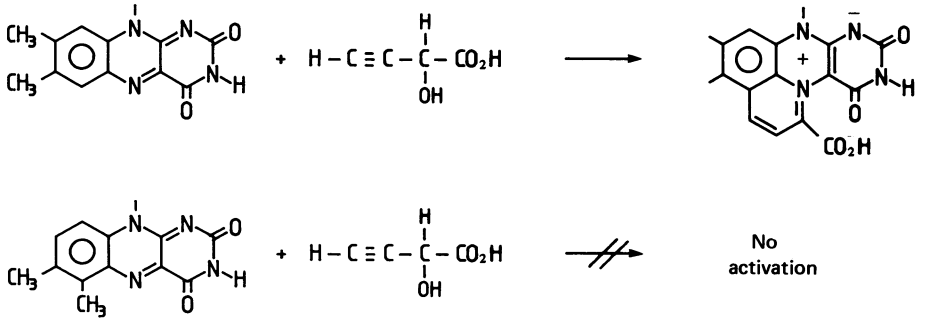


Fig. 15. Use of isoflavin as a mechanistic probe

Formation of a 5,6-cyclic adduct of α-hydroxybutynoate with D-lactate dehydrogenase, and lack of reaction when the normal FAD of the enzyme is replaced with iso-FAD.

PHOTOAFFINITY LABELS

The established method for creating photoaffinity labels by inserting the azido function into a chromophore [79] relies on the generation of a highly active nitrene from photodecomposition of the azide:

$$R\text{–}N_3 \xrightarrow{h\nu} R\text{–}N\!> + N_2$$

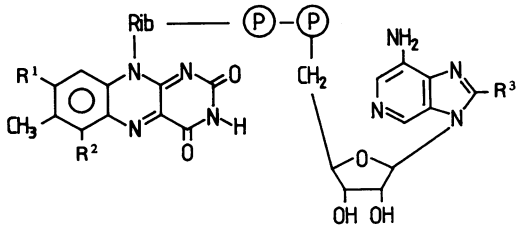
$$R\text{–}N\!> + \text{protein–X–Y} \rightarrow R\text{–}NY\text{–X–protein}$$

The nitrene then reacts by insertion into a protein residue and leads to covalent labelling. This method should be particularly well suited to the flavin coenzyme since the latter has transitions in the visible/near-u.v., which do not overlap with the absorption of amino acid residues. Therefore the danger of photochemical destruction of the protein should be minimized. The azido group has been introduced into three positions of flavin coenzymes (Fig. 16).

Koberstein has synthetized 8-azido(adenine)-FAD, incorporated it into apo-D-amino acid oxidase, and shown that upon illumination the flavin is covalently bound to the protein, still retaining some activity [80]. Although no effort was made to identify the protein residue(s) involved, it is likely that this reagent will label the adenosine-binding site.

8-Azidoflavin derivatives have been synthesized from 8-amino- or 8-fluoroflavins [81], and have been found to be extremely photoactive; they can be handled only under careful exclusion of light. Substantial covalent labelling as a result of light irradiation was found with glucose oxidase and with riboflavin-binding protein, while with Old Yellow Enzyme, flavodoxin, and D-amino acid oxidase only small extents of covalent attachment to the protein were observed [81], in agreement with the results obtained with other chemically reactive flavins, summarized in previous sections.

In contrast to the 8-azidoflavins the recently synthesized 6-azidoflavins are less light sensitive, thus simplifying their handling [21]. Their photoactivity, however, is sufficiently high as to yield appreciable covalent reaction with several proteins (V. Massey, S. Ghisla & K. Yagi, unpublished work). Thus 6-azido-FAD is bound by D-amino-acid oxidase with an affinity comparable to that of normal FAD. The effect of light irradiation on this complex is shown in Fig. 17. The final product has a spectrum which is similar to that of 6-amino-FAD bound to the same enzyme; it is, however, not 6-amino-FAD. In contrast with the latter, the product



(*a*) $R^1 = -N_3$, $R^2 = -H$, $R^3 = -H$
(*b*) $R^1 = -CH_3$, $R^2 = -N_3$, $R^3 = -H$
(*c*) $R^1 = -CH_3$, $R^2 = -H$, $R^3 = -N_3$

Fig. 16. Structure of azido-flavins which have been used as photoaffinity labels

(*a*), 8-N_3-FAD; (*b*), 6-N_3-FAD; (*c*), 8-N_3 (adenine)-FAD.

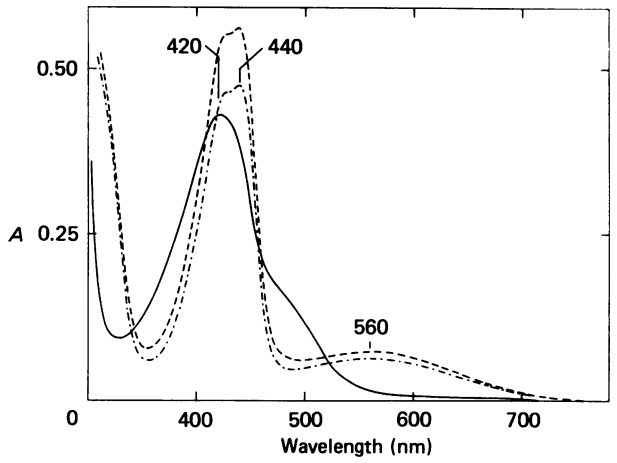


Fig. 17. Covalent attachment of protein and flavin on light irradiation of 6-N_3-FAD D-amino acid oxidase

Solid curve, 6-N_3-FAD enzyme before light; broken curve, after light irradiation; broken/dot curve, after extensive treatment with KBr to remove non-covalently bound flavin (V. Massey, S. Ghisla & K. Yagi, unpublished work).

is not reducible by substrate, it does not bind benzoate, and, most importantly, the chromophore is not released from the protein under conditions under which normal FAD or 6-amino-FAD are released quantitatively. This suggests that upon photoirradiation the flavin is attached to the protein via the position (6) nitrogen. Unfortunately, however, all conditions which denature the enzyme were found to destroy also the covalent linkage, thus rendering an analysis of the product difficult. In view of the properties of the product it is likely that the modification probably involves either the protein function involved in binding of substrate or that which functions in abstraction of the substrate α-hydrogen (see [3]).

CONCLUSIONS

The type of information about accessibility to the various positions of flavin bound to specific proteins is summarized in Table 1. The validity of the technique is supported by agreement of the results with the structures of the three flavoproteins in this list which have been determined by X-ray crystallography, flavodoxin [31], glutathione reductase [30], and *p*-hydroxybenzoate hydroxylase [29]. Indeed the solvent accessibility to the 8-position of the enzyme bound flavin in *p*-hydroxybenzoate hydroxylase had been determined successfully [9] before the crystal structure, and revealed that conformational changes occur in the protein, at least around the flavin 2-position, when substrate is added [44].

An inspection of Table 1 reveals several interesting trends. All flavoproteins so far studied (including several not included in the Table) which react with pyridine nucleotides as one of their substrates are exposed to solvent at the flavin 8-position. In both glutathione reductase and *p*-hydroxybenzoate hydroxylase [31] the three-dimensional structures show a channel lying over the *re*-face of the flavin, which permits access of the

Table 1. Correlations of structure and function among flavoproteins

Flavoprotein	Radical[a]	Topography[b] at position: 2	4	5	6	8	8-Mercapto-flavin[c]	6-Hydroxy-flavin[d]	6-Mercapto-flavin[e]	8-Hydroxy-flavin[f]	References
General acyl-CoA dehydrogenase											
−Substrate	Blue	Exposed	–	(Exposed)	–	Buried	Resolved benzoquinoid	Neutral (pK 8.7)	–	–	78,87
+Substrate	Red	–	–	(Exposed)	–	Buried	Resolved benzoquinoid	anion (pK < 7)	–	–	
D-Amino acid oxidase	Red	Buried	Exposed	Exposed	Partly buried	Exposed	Resolved benzoquinoid	Anion (pK < 5)	Anion (pK < 5)	–	86,44,38,46,*g*, 43,28
L-Lactate oxidase	Red	Buried	Exposed	Exposed	Partly buried	Buried	Resolved benzoquinoid	Anion (pK < 5)	Anion (pK < 5)	Anion (pK < 4)	44,38,46,*g*,43,28
Glucose oxidase	Red/blue pK 7.3	Exposed?	–	–	–	Buried	Resolved benzoquinoid	Anion (pK 5.6)	– –	– –	86,44,46,43.28
Melilotate hydroxylase											
−Substrate	None	–	(Exposed)	(Exposed)	–	Exposed	Smooth benzoquinoid	–	–	–	
+Substrate	None	–	(Exposed)	(Exposed)	–	Exposed	Thiolate	–	–	–	*h*,44,38,43,28
p-Hydroxy-benzoate hydroxylase											
−Substrate	Red/blue pK ~ 7 (unstable)	Exposed	Exposed	(Exposed)	–	Exposed	Thiolate	pK 6.9	– –	– –	
+Substrate	Blue (unstable)	Buried	Partly buried	(Exposed)	–	Exposed	Smooth benzoquinoid	–	–	–	*j*,44,38,43,28
Ferredoxin–NADP reductase	Blue	Buried	–	(Exposed)	–	Exposed	Smooth benzoquinoid	Neutral pK ≧ 10	–	–	86,77
Glutathione reductase	Red	Buried	Buried	(Exposed)	Exposed	Exposed	Smooth benzoquinoid	Binds poorly pK ~ 7.2	Anion pK < 4.5	– –	32,*k*,90
Flavodoxin (*M. elsdenii*)	Blue	Buried	Buried	(Buried)	Exposed	Exposed	Smooth benzoquinoid	Neutral pK ~ 9	Neutral pK 6.4	Neutral pK 6.1	86,44,38,43,*g*,28
Old Yellow Enzyme	Red	Buried	Exposed	(Exposed)	Exposed	Exposed	Smooth benzoquinoid	Anion pK 5.7	Anion pK not determined	Neutral pK 6.2	86,44,38,43,*g*,*h*, 88,28
Riboflavin-binding protein	Blue	Exposed	Buried	(Buried)	Buried	Buried	Neutral (pK > 8)	Neutral (pK > 8)	Neutral (pK > 8)	Neutral pK ~ 9	89,44,38,*g*,43,28

[a] With free flavin the pK of the radical is 8.5 [6]; the neutral species is blue and the anion is red [86].
[b] When parentheses are used the ascription is presumed from other studies, and not shown directly with chemically reactive flavins.
[c–f] The pK values of the free flavins are: [c]3.8 [27], [d]7.1 [18], [e]5.9 [21], [f]4.8 [19].
[g] V. Massey, S. Ghisla & K. Yagi, unpublished work.
[h] L. M. Schopfer, unpublished work
[j] R. Anderson, V. Massey & L. M. Schopfer, unpublished work.
[k] V. Massey, S. Ghisla & G. Schulz, unpublished work.

pyridine nucleotide to the flavin. It is tempting to speculate that this may be a feature common to all flavoproteins reacting with pyridine nucleotides, a possibility made more likely by the recent discovery that the stereospecificity of hydride transfer between reduced pyridine nucleotides and six different flavoproteins (glutathione reductase, mercuric reductase, thioredoxin reductase, *p*-hydroxybenzoate hydroxylase, melilotate hydroxylase and anthranilate hydroxylase) all involve the *re*-face of the enzyme-bound flavin (D. J. Manstein, E. F. Pai, L. M. Schopfer & V. Massey, unpublished work).

Another generalization which appears to hold with flavoenzymes of the oxidase class is that they all are exposed to solvent around the flavin N(5)–C(4)=O locus, that they all stabilize the anionic semiquinone and the anionic fully reduced flavin, and that they give substantial stabilization of the anionic forms of 6-hydroxy-, 6-mercapto-, and 8-hydroxyflavins, i.e. the pK of the flavin is lowered on binding to the protein. These same proteins also stabilize without exception the blue benzoquinoid form of 8-mercaptoflavins. All these properties can be ascribed to the stabilizing influence of a protein positively charged residue in the neighbourhood of the flavin N(1)–C(2)=O locus [28,36,46,47].

The results shown in Table 1 also suggest a possible trivial explanation for the longtime puzzle of the different reactions and reactivities of different classes of flavoproteins with molecular oxygen. It is now well documented that all flavoenzymes of the hydroxylase/mono-oxygenase class in their reduced form react with O_2 to give a flavin C(4a)-hydroperoxide species, which is stabilized in different ways in different enzymes (see [82] for a recent review). It is generally agreed that the formation of the hydroperoxide proceeds by a radical mechanism, as shown below:

$$Fl_{red.}H^- + O_2 \leftrightarrow [FlH^{\cdot}\uparrow\downarrow O^{\cdot}{}_2^-] \leftrightarrow [FlH^{\cdot}\uparrow\downarrow O^{\cdot}{}_2^-] \leftrightarrow FlH\text{–}OO^-$$

First a one-electron transfer occurs to form a flavin radical-superoxide pair having parallel spins. After spin inversion, which is presumably the rate-limiting step, the biradical pair collapses to form the flavin C(4a)-hydroperoxide. In model studies, the pair of radicals, $FlH^{\cdot}$ and $O_2^{\cdot-}$, generated by pulse radiolysis, has been shown to collapse into the hydroperoxide in the μs time range ($k = 7.8 \times 10^8\ M^{-1} \cdot s^{-1}$) and subsequently to decay to oxidized flavin and peroxide in the pH range 6–7 at a rate of 260 s^{-1} [83]. In flavoproteins the formation of the 4a-hydroperoxide can only occur when the flavin C(4a)-position is exposed to solvent. From Table 1, it would appear that both oxidases and hydroxylases meet this criterion, but electron transferases such as flavodoxin do not. In such a case, there would seem to be no alternative to the radical pair dissociating into the free radical species, flavoprotein semiquinone and $O_2^{\cdot-}$, the well-documented products of reaction of reduced flavodoxin and O_2 [37,84]. On the other hand there would seem to be no steric barrier to oxidases forming the flavin C(4a)-hydroperoxide as an intermediate, but such intermediates have perhaps escaped detection because of the inherent instability of the hydroperoxide, i.e. that some positive stabilizing effect by the protein has to exist for the mono-oxygenases where the hydroperoxide is readily detected. That oxidases can in fact form a hydroperoxide intermediate has been shown by pulse radiolysis experiments with glucose oxidase, where flavoprotein semiquinone and $O_2^{\cdot-}$ were shown to collapse rapidly to the hydroperoxide ($k \sim 6 \times 10^8\ M^{-1} \cdot s^{-1}$) and then decay to oxidized enzyme and H_2O_2 with a rate constant of $\sim 300\ s^{-1}$ [85]. Under the same conditions there was no detectable formation of flavin hydroperoxide from flavin semiquinone and $O_2^{\cdot-}$ with flavodoxin.

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