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Flavoenzyme Structure and Function

Approaches Using Flavin Analogues

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1. Introduction

Flavoenzymes are redox proteins that catalyze a wide diversity of biological reactions ranging from O_2 activation, to aromatic hydroxylations, dehydrogenations, and reactions in which they can accept or donate one or two electrons. In addition, they can fulfill structural and regulatory roles. This diversity is due to the fine tuning of the reactivity of the isoalloxazine ring of the flavin coenzyme by specific interactions with the protein moiety on the particular enzyme it complexes with. Methods to provide specific information on these interactions have relied on three approaches:

1. Determination of the three-dimensional structure of the flavoenzyme by X-ray diffraction techniques.
2. Spectroscopic probes of the protein influence on flavin structure by techniques such as nuclear magnetic resonance (NMR), resonance Raman, fluorescence, electron paramagnetic resonance (EPR), and circular dichroism (CD) spectroscopies.
3. Replacement of the native flavin coenzyme with suitable flavin analogues designed to ask specific questions regarding the reactivity, the accessibility, and the mode of specific interactions with the protein.

The first approach is labor intensive, requires the enzyme to crystallize in a form that diffracts with high resolution, and requires a knowledge of the protein's amino acid sequence for interpretation of the electron density maps. The second approach requires the synthesis of isotopically-labeled flavins (for NMR, resonance Raman, and EPR spectroscopies) to facilitate interpretation

of spectral data. These techniques also require reasonably large quantities of purified flavoenzyme (especially for NMR analysis) and access to specialized instrumentation and to investigators knowledgeable in these techniques. The third approach provides a good deal of unique information on the environment and reactivity of the flavin in its binding site, does not require large quantities of enzyme, and does not necessarily require access to highly specialized instrumentation. The major requirements are that the flavoenzyme is amenable to reversible resolution of the flavin coenzyme (*see* Chapter 11) and reconstitution of the apoenzyme with suitable flavin coenzyme analogues. This approach does require the availability of the riboflavin form of these analogues, which, in general, have to be obtained by chemical synthesis. Furthermore, the riboflavin analogues have to be enzymatically converted to either coenzyme form (FMN or FAD). This is conveniently done enzymatically with the *Brevibacterium* FAD synthetase system *(1)*. Once the FAD analogue is prepared, it can readily be converted to the FMN level by treatment with nucleotide pyrophosphatase.

Here, we will outline the basic approaches which can be applied to the study of those flavoenzymes containing noncovalently bound flavin coenzymes. Those containing covalently-bound flavins have not been amenable to this approach for obvious reasons. Recent developments in the area of covalent incorporation of flavins into members of this class of flavoenzymes (e.g., *p*-methyl cresol hydroxylase *(2)* and monoamine oxidases *(3)*) should provide the technology for similar types of approaches in this class of flavoproteins.

2. Historical Perspective

Flavin coenzymes are comprised of the redox-active isoalloxazine ring, a ribityl side chain, and (for FMN) a 5'-terminal phosphate ester or (for FAD) a pyrophosphate linkage of FMN with an aminomonophosphate (AMP) moiety (**Fig. 1**). Each of these moieties is intimately involved in binding the coenzyme to its site on the apoenzyme. Until recently, the ribityl side chain and the level of phosphorylation was thought only to be involved in binding interactions and that all of the chemical reactivity of the flavin in catalysis resided only in the ring system. Although this view may be true for the vast majority of flavoenzymes, it is not universally valid with the recent discovery of the required participation of the 2' OH group of the ribityl side chain in acyl-CoA dehydrogenase catalysis *(4)*. However, the bulk of informative work with modified flavins has been performed with ring-modified analogues.

The synthetic procedures for synthesis of ring-modified riboflavin analogues were developed mainly in Lambooy's and Hemmerich's laboratories (**refs. 5–7** for synthetic procedures). Approaches developed to monitor the reactivities of

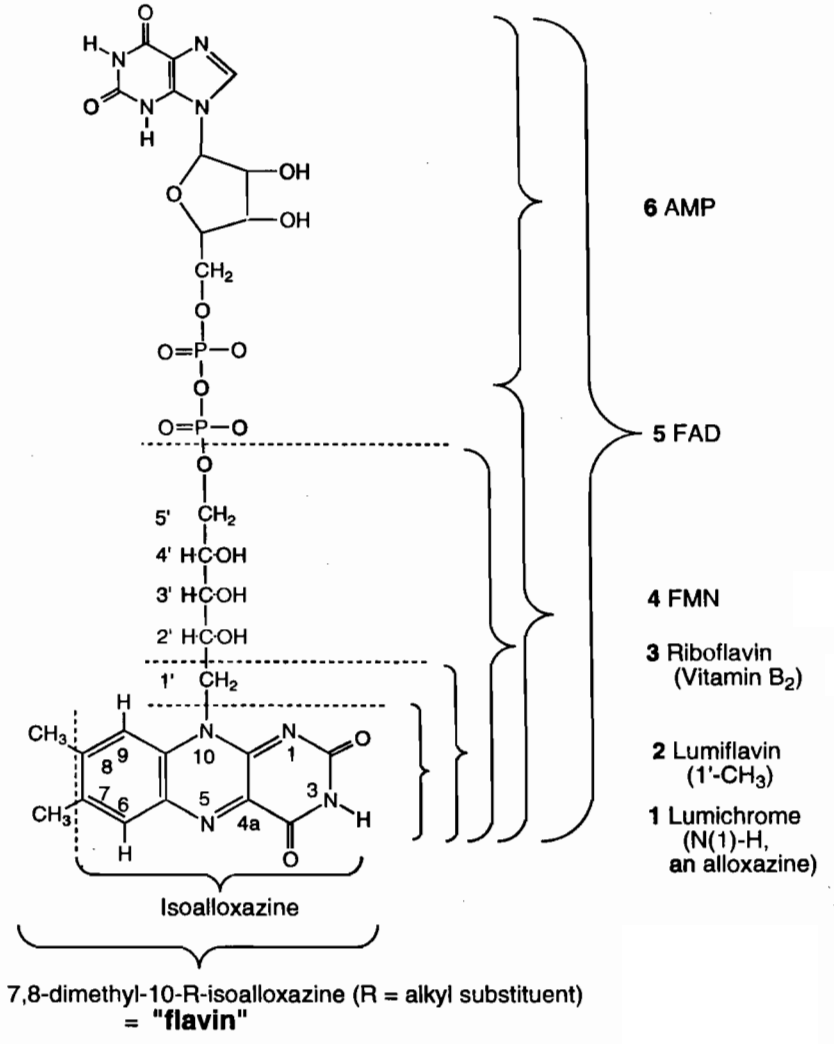

Fig. 1. Structures of various derivatives of the flavin. Note that the term "flavin" refers to an isoalloxazine molecule carrying two methyl groups at the positions 7, and 8, and a further substituent at position N(10). The ring numbering shown is that approved by the International union of Pure and Applied Chemistry. Different numbering systems are to be found in the older literature.

the flavin analogues when bound to the protein were developed in Massey's laboratory with the collaboration of one of the co-authors (S.G.). The enzymic conversion of riboflavin analogues to their FAD and FMN coenzyme forms is described in detail in *(8)*.

3. Rationale and Basic Considerations.

The information that can be obtained from studies with modified flavins can be subdivided into three distinct topics of study:

1. *Molecular environment of the isoalloxazine ring system, and accessibility* (study of the interaction of the flavin with its protein environment, specifically with amino acid groups, and of the regulation or prevention of access to flavin sites by reactive molecules.
2. *Mechanistic studies* (approaches aimed at the differentiation of catalytic mechanism reaction types).
3. *Modulation of the oxidation-reduction potential* (studies aimed at the understanding of the fine tuning of the flavin reactivity via its redox potential).

3.1. Flavin Interactions and Accessibility

As mentioned above, the ribityl side chain of the flavin serves in anchoring the catalyst to the protein. This chain, in both FAD and FMN, has been found from crystallographic studies on a number of flavoenzymes to be buried, at least partially, inside the protein. The interactions of the coenzyme side chain with the protein contribute the majority of the binding energy to the energetics of FMN or FAD binding to their respective apoenzymes. The 7,8-dimethylisoalloxazine moiety, i.e., the chromophoric part of the flavin, is an amphoteric molecule in that its xylene aromatic ring is lipophilic, while the pyrimidine subnucleus is hydrophilic. The discussion of accessibility/reactivity of the flavin can thus be subdivided into those of the pyrimidine ring and of the benzenoid domains (*see* **Fig. 2**) Already a superficial inspection of the structures (**Figs. 1** and **2**) make it clear that the pyrimidine ring will undergo specific interactions (i.e., hydrogen bonding) with protein functional groups. The mode of such interactions, as well as the accessibility of the pyrimidine moiety has been probed in various ways:

1. Position N(3)-H can be altered by alkylation (**Fig. 3**), and the size and properties of the N(3)-substituent can be varied. This approach will verify if the N(3)-H group undergoes an important interaction. 3-Methyl-FMN and FMN-3 acetate have been used for this purpose *(9)*. In general, most flavoproteins do not appear to be very tolerant of substantial changes at the isoalloxazine N(3) position.
2. The C(2)=O carbonyl, which is part of the pyrimidine amidine system, has been replaced with =N-R and =S. This opens two types of approaches. On the one hand the bulkier sulfur atom and its different chemistry compared to oxygen, can disturb the strength and geometry of H-bonds. These effects can to some extent balance their effects on the flavin ring in that the stronger electronegativity of the sulfur can compensate for the weaker H-bonds that it forms. The thiocarbonyl function, on the other hand, is a much better nucleophile and electrophile compared to the essentially unreactive C(2)=O group. Thus, 2-thioflavins will react

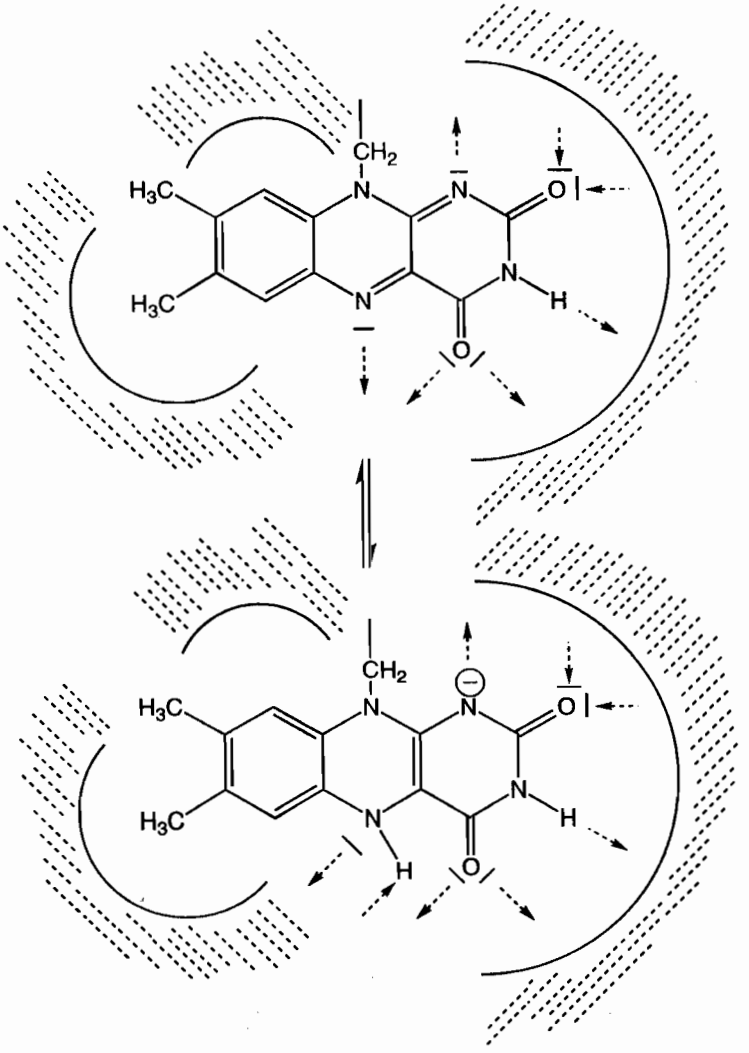

Fig. 2. Modes and positions of interaction of the various flavin functional groups with the protein. The latter is symbolized by the shaded areas. Arrows denote the possibility to form donor (→) or acceptor (←) hydrogen bonds. The open areas indicate positions at which accessibility has been found in a majority of cases. This does not necessarily apply to all flavoproteins.

with a variety of alkylating agents and are prone to oxidation ***(10)***. The (2)S group can also easily be substituted with amines and other nucleophiles, which are listed in **Fig. 4**. Upon determination of the basic reactivity of the (2)=S func-

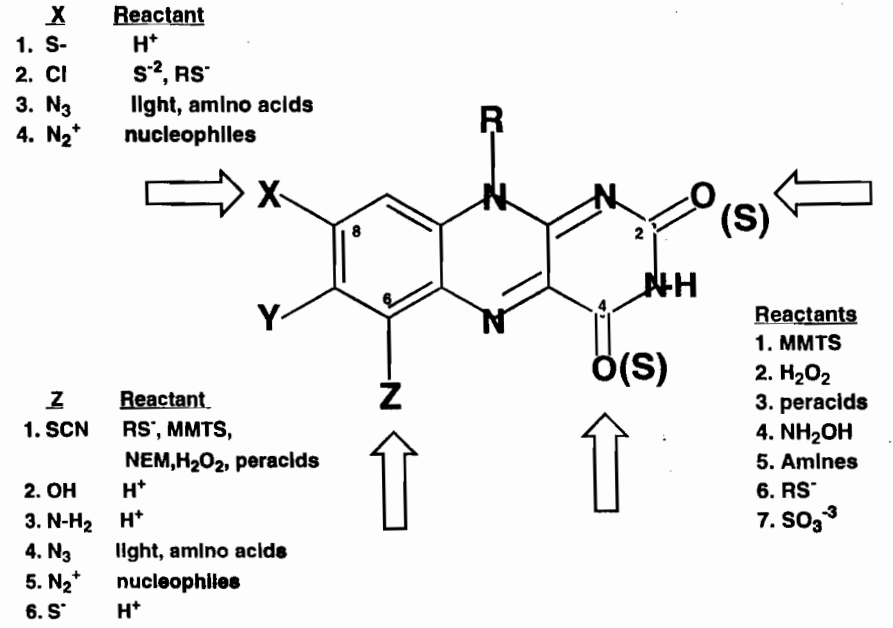

Fig. 3. Overview of the functional groups on the flavin ring that are amenable to chemical analysis by either modification or substitution. The arrows indicate the positions for which most of the studies have been done. This does not exclude that other reactive groups could be introduced in the flavin ring system. Study of the reactivity with the reactants shown can be used to monitor either the accessibility of the specific flavin position when bound to a protein, or its (modified) reactivity.

tion with a given reactant, the rate of reaction of the enzyme bound (2)=S flavin will reflect the degree of accessibility of this position from the bulk solvent. The approach has been implemented (e.g., with *p*-hydroxybenzoate hydroxylase) reconstituted with 2-thio-FAD and H_2O_2 as reactant ***(11)***.

3. The same type of approach can be implemented at position C(4)=O. In general, it should be stated that modifications at these positions are more labile, than the similar C(2)=O position discussed above. Two publications deal with this topic ***(10,12)***. One particularly useful property of 4-thioflavins should be mentioned. The differentiation between the neutral and the anionic forms of reduced flavins bound to various enzymes is, in general, very difficult due to the spectral similarity of the two species, and the lack of particular features. In contrast, the reduced species of 4-thio-flavins exhibit well resolved absorption spectral bands with the maxima at 425 nm for the anionic and 485 for the neutral forms ***(10)***.

The (middle) pyrazine ring of the flavin system comprises the main locus of chemical reactivity, i.e., the positions C(4a)-N(5), which are involved in elec-

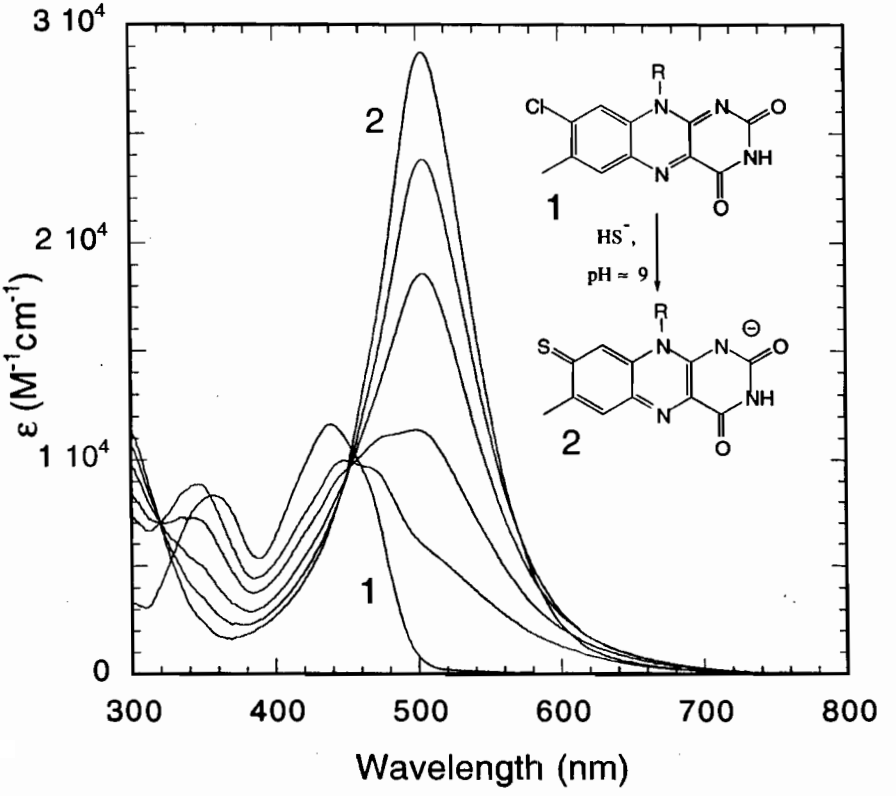

Fig. 4. Spectral changes accompanying the conversion of 8-Cl-flavin into 8-thioflavin (in its anionic form). The reaction was carried out anaerobically at 25°C, and in the presence of ≈10 m*M* of the reactant.

tron donation and uptake. Accessibility to these positions is to be found in those enzymes which carry out chemistry with a substrate (as opposed to the cases where the flavin exhibits a structural or electron transfer role). Accessibility to position C(4a) appears to be crucial for the formation of reactive oxygen hydroperoxides. Strategies to probe this specific point are difficult to conceive and have not yet been implemented.

The xylene ring is regarded as being "unreactive," mainly because there are no cases in which chemical events resulting from interaction with substrate have been found. Often this moiety is inserted into hydrophobic protein pockets. However, an unexpected feature has emerged: position C(8)-CH_3 appears to be near the protein surface in many flavoenzymes, in which it has no (detectable) function. In several cases it is freely accessible to bulk solvent, while other parts of the molecule are not. Massey's group has studied this aspect

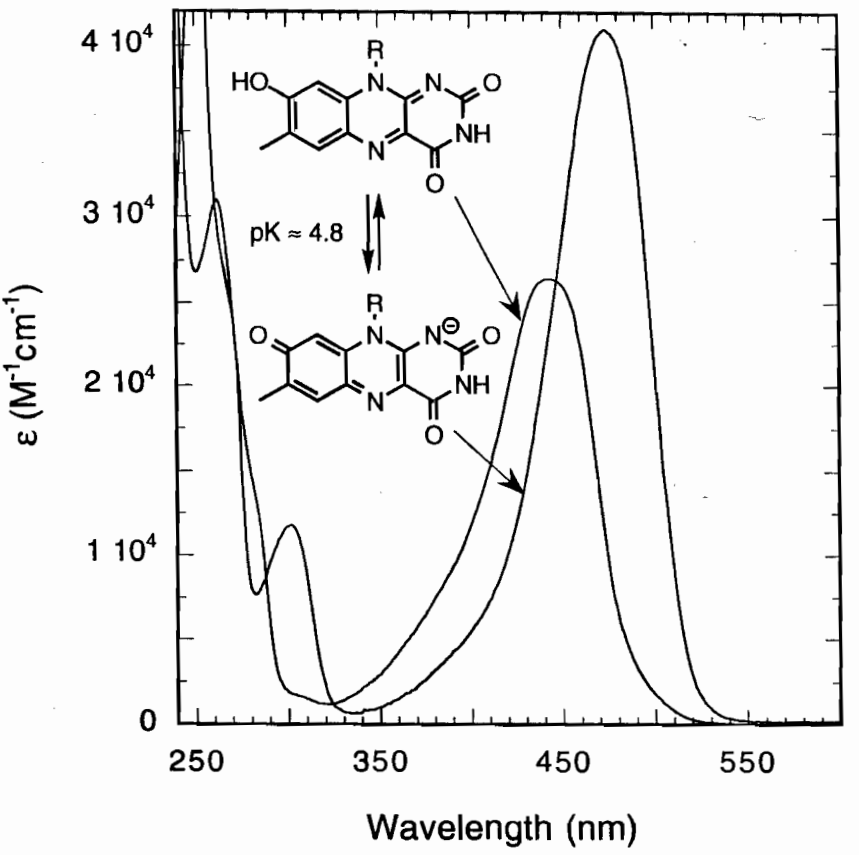

Fig. 5. Spectral changes accompanying ionisation of 8-OH-flavin. Such changes can be used to monitor e.g., the charge distribution at the active center on an enzyme, or p*K* shifts induced by the latter.

using the reactivity of 8-mercapto-flavins bound to various enzymes with alkylating electrophiles in the presence and absence of substrate *(13)*. Accessibility to position C(8)-CH_3 can also be probed using 8-Cl-8-nor-flavins by following its reaction with sulfur nucleophiles. These approaches are easily implemented experimentally since they are based on the large spectral changes accompanying the conversions. Two examples are shown in **Figs. 4** and **5**, where the spectral effects accompanying the conversion of 8-Cl-8-nor-flavin into the (8)-S-anion, **(Fig. 4)**, and that of the latter into its neutral form are shown (**Fig. 5**) Note that the absorption spectrum of the 8-SH and 8-S-alkyl flavins are very similar.

The flavin nucleus is also a sensitive tool for studying the environment surrounding it.

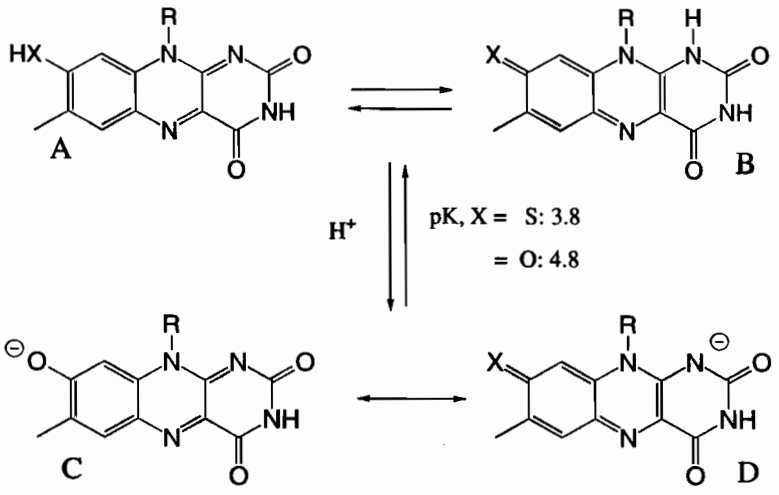

Fig. 6. Possible forms of 8-substituted flavins. Neutral species can exist either in the "phenolic" form (A), or in the "paraquinoid" form (B). These are spectrally distinct species. Ionisation leads to a delocalized system in which the charge can be delocalized in the pyrimidine moiety as shown by the canonical structure (D), the one which predominates in free solution. Stabilization of the "phenolate" structure (C) can be induced by binding to a protein active site.

The shape of the absorption spectrum of normal flavin is somewhat dependent on the solvent, the differences probably reflecting the relative intensities of single vibronic transitions, and being best detected by difference spectroscopy. Such a study has been presented in 1973 by Massey's group *(14)* in which also the temperature dependence of the flavin spectrum has been reported.

Binding of the flavin chromophore can be accompanied by very large perturbations of the spectrum as, e.g., in the case of cholesterol oxidase, where two members of the same family exhibit very different absorbance spectra *(15)*. The direct interpretation of such effects is, however, very difficult, if not impossible in the case of flavoproteins. More information about the protein environment can be obtained using flavins in which the charge distribution is very different. This is the case with 8-substituted-8-nor-flavins, where the substituent is -NR_2, -SH, or OH. These molecules can exist in two canonic states as represented in **Fig. 6**, and where the charge (or the X-H bond) is either at position N(1) or on C(8)-X. Obviously these species are spectrally very different. With neutral 8-SH and 8-OH, the predominant form is a "phenolic" one, as shown on the left hand side on **Fig. 6**. The spectra are accordingly similar to those of normal flavins with maxima in the 450 nm region (**Fig. 7**). With 8-NH_2-8-nor-flavins, and with the anionic forms of 8-S-, and 8-O-8-nor-flavins the paraquinoid structure shown in **Fig. 6** (right hand side structure)

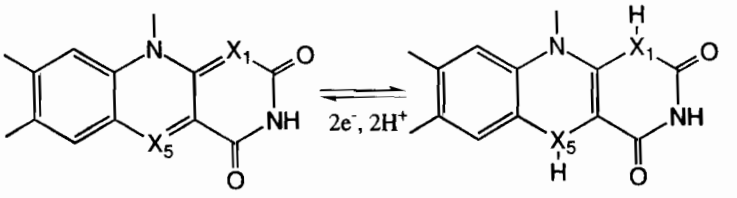

Fig. 7. Positions at which an isoelectronic exchange of "N" for "C-H" has been implemented in the flavin system.

predominates. This has been shown based on model work in which the respective forms can be blocked by alkylation *(16,17)*. It should be pointed out, that in the anionic forms just named, the negative charge is located predominantly at the N(1)-C(2)=O locus. These flavins are thus, from the point of view of charge distributions, analogues of *reduced* flavins, where the negative charge also sits at the same location. The stabilization of the reduced flavin negative charge is an important factor in determining the redox potential of the couple, in that a stabilization, corresponding to a pK decrease, is equivalent to an increase of redox potential. Such flavins have thus been employed in the detection of a negative charge near N(1) e.g., in the L-lactate oxidase, the glycolate oxidase family *(18)*, or with glutathione reductase, where an induced dipole brings about the same effect *(19)*. The opposite, i.e., the destabilization of the paraquinoid form of anionic 8-S- or 8-O-flavins in favor of the phenolic mesomeric forms occurs with flavodoxins *(17)*, which is, in turn, nicely correlated with the low redox potential of these proteins. **Figure 7** depicts an example of the effect of the 8-OH ionization on the chromophoric system of 8-hydroxy-8-nor-flavin.

3.2. Flavin Analogues as Mechanistic Probes

The outstanding property of the flavin ring is its chemical versatility which leads to a capacity to carry out differing reactions by intrinsically different mechanisms. This, in turn, can make the determination of a specific mechanistic variant very difficult. For answering this question flavin analogues can be tailored to carry out only specific reactions, while not allowing others. The modifications which have been most fruitful in this respect are based on the isoelectronic substitution of N with a C-H, which has been implemented at the flavin positions N(1) and/or N(5). The structures of these analogues are shown in their corresponding oxidized and 2 e- reduced forms (**Fig. 8**).

Four general questions can be addressed using these models; based on the following diversities:

1. Formation of acceptor H-bridges (*see* also **Fig. 2**). This is possible with *N*-H but not with C-H.

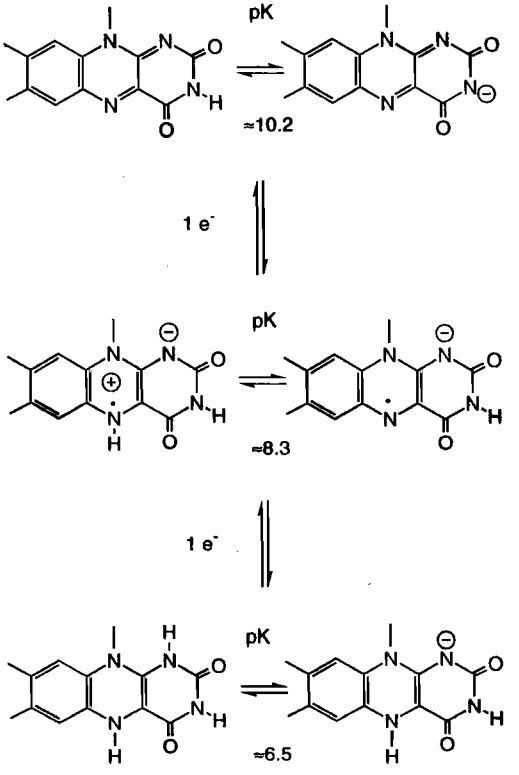

Fig. 8. Relevant structures of oxidized, "half-reduced" (semiquinone) and "fully reduced" (dihydro) flavins, and corresponding ionisations of biological relevance.

2. Formation of donor H-bridges: Possible with the N lone pair, not with C-H.
3. Occurrence of formation/rupture of a *N*-H bond during catalysis, and stereospecificity. The *N*-H bond is intrinsically labile, the C-H bond is stable. This difference can lead to mechanistic differentiations (e.g., in a transition state and in analysis of product stereospecificity).
4. Mode of rupture of a *N*-H bond during catalysis: Radical states are formed with much lower energy on N (and thus are more stable) than on a C-H center. Consequently, 5-deaza-5-carbaflavins have proven to be inactive in 1e- electron transfer processes.
5. Occurrence of covalent intermediates in catalysis. Such species would be expected to be stable when involving a C-C bond, as opposed to labile (i.e., catalytically viable) for the *N*-C case (one C originating from the flavin, the second from a reactant).

These properties have led to the study of 5-deaza-5-carba-FMN or -FAD as replacements of the normal flavin coenzymes in flavoenzymes and have proven to be mechanistically valuable flavin analogues. As stated above, this analogue

is an obligatory two-electron acceptor/donor due to the unfavorable energetics involved in (transient) semiquinone formation. A survey of the literature shows the flavin to be functional in H^- (hydride) transfer reactions. Based on the reactivity with pyridine nucleotide reductants, this analogue has been suggested (probably in a strong oversimplification) to be a "flavin-shaped pyridine nucleotide analogue" ***(20)***. No activity is observed in systems expected to occur by one-electron transfer steps and no evidence for any covalent intermediates in flavoenzyme catalysis have been observed. The reduced flavin analogue is unreactive with O_2 which is expected mechanistically, as the reaction with normal flavin hydroquinone involves a one-electron transfer to form $O_2{\cdot}^-$-flavin semiquinone radical pair as the initial reaction step ***(21)***.

The 5-deaza-5-carbaflavin analogue, at the FMN level, was first introduced by Edmondson et al. ***(22)*** for studies with flavodoxin. These initial studies demonstrated that it binds to the protein but that it does not form a stable semiquinone as found with other FMN analogues with N at the 5-position. The analogue is capable of accepting H^- from pyridine nucleotides in several nicotinamide-adenine-dinucleotide phosphate (NAD(P)H) dependent flavoenzyme reductases or from H^- donors such as BH_4^-. Hydride donation to 5-deaza-5-carbaFAD has been demonstrated with medium chain acyl-CoA dehydrogenase, which catalyzes the αβ-dehydrogenation of acyl CoA thioesters ***(23)***. Also of basic importance are the observations of incorporation of substrate-derived H-label into the 5 position of 5-deaza-5-carbaflavin coenzymes (suggesting a H^- mechanism) with the following enzymes: D-amino acid oxidase ***(24,25)***, lactate oxidase ***(26)***, and flavocytochrome b_2: lactate dehydrogenase ***(27)***. All these enzymes have long been thought to function by a H^+ abstraction from the α-C-H of the substrate as the mechanism of C-H bond cleavage. These observations would be in keeping with recent structure-activity studies of D-amino acid oxidase ***(28)*** and L-lactate oxidase ***(29)*** which also are compatible with a H^- mechanism. Furthermore the 3-dimensional crystal structure of D-amino acid oxidase ***(30)*** and the properties of mutants of the same enzyme in which a potential H^+-abstracting base is absent, provide additional evidence in support of a H^--transfer mechanism. An alternative possibility that requires consideration (and further study) is the suggestion that substitution of 5-deaza-5-carbaflavin for the normal flavin enforces a change in mechanism and thus a different mode of C-H bond cleavage ***(26,31)***.

The 5-deazaflavin analogue 8-hydroxy-8-nor-5-deaza-5-carbaflavin has been shown ***(32)*** to be a valuable probe to determine the stereochemistry of H^- transfer from pyridine nucleotide to the enzyme-bound flavin. Five different pyridine nucleotide-dependent enzymes (glutathione reductase, mercuric reductase, *p*-hydroxybenzoate reductase, melilotate hydroxylase, and anthranilate hydroxylase) have all been shown to be reduced at the *re*-face of the

bound flavin (by H^- transfer) in agreement with predictions from crystal structures of two of the enzymes tested *(31)*. In the case of medium chain acyl-CoA dehydrogenase transfer of the substrate β-H also occurs onto the flavin *re*-face *(32)*.

5-Deaza-5-carbaflavins have been particularly useful in the assessment of the oxygen reactivity of reduced species *(33)*. 1-Carba-1-deaza-FMN has been used successfully to refute the proposal *(34)*, that in bacterial luciferase the emitter is an N(1) protonated flavin cation. This conclusion was based on the finding that the 1-carba-1-deaza-FMN luciferase complex is competent in oxygen activation and light emission, but cannot protonate at position 1 *(34)*.

Another mechanistic approach where 8-substituted flavin analogues have been profitably used is in the application of linear free energy relationships for testing proposed mechanisms. Two types of enzymatic mechanism have been successfully tested. With bacterial luciferase the mode of generation of the excited state (light-emitting species) has been shown to proceed via a process in which charge is donated from the reduced flavin to the reactant subspecies, probably the aldehyde hydroperoxihemiacetal *(35)*. A series of 8-substituted FMNs was used with bacterial luciferase and the rate of light emission decay was correlated with the $1e^-$ oxidation potentials of a corresponding series of 8-substituted lumiflavin models. The results were concluded to be compatible with a CIEEL type mechanism. With *p*-hydroxybenzoate hydroxylase the reactivity of flavin 4a-hydroperoxides in catalytic substrate monooxygenation was correlated with the p*K*a of the 4a-OH products perturbed by the inductive electronic effect from the 8-substituent using various 8-substituted flavins *(36)*. This p*K*a was assumed to be linearly correlated with the Eox of the corresponding oxidized flavin. Similar 8-substituted flavin models have been used in Bruice's laboratory *(37)* to probe an electrophilic aromatic substitution mechanism in flavin-mediated hydroxylations. Indeed the flavin-4a-hydroperoxide was deduced to be the hydroxylating agent since the rate of hydroxylation is correlated to the inductive effect of the 8-substituent on the flavin which modulates the p*K* (and stability) of the resulting flavin-4a-hydroxy anion.

3.3. Oxidation-Reduction Potentials of Flavin Analogues

Because flavoenzymes catalyze either one- or two-electron transfer steps in catalysis, the ability to alter the energetics of these reactions by modification of the flavin ring is a valuable mechanistic probe. The protein environment of the bound flavin can modify the flavin redox potential in three ways: (1) through donating or accepting H-bonding to the pyrazine and pyrimidine rings and to N(5) (*see* **Fig. 2**), (2) by suitable placement of charge proximal to flavin ring positions that develop a charge on one- or two-electron reduction; the charge(s) can be positive or negative, thus stabilizing or destabilizing the (transient) species, and (3) by insertion of (parts) of the flavin ring(s) into

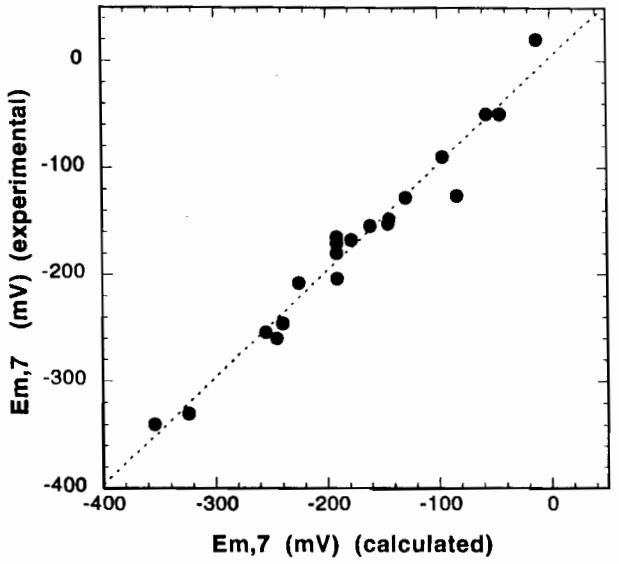

Fig. 9. Correlation of calculated and experimental redox potentials of the flavins listed in Table 2. The correlation has a slope of 1. See text for further details.

environments of varying dielectric (hydrophobic/hydrophilic) can have similar effects. The normal oxidized flavin ring is electrically neutral but develops charges on semiquinone formation to either the zwitterionic neutral (blue) or the anionic (red) semiquinone (pK = 8.4) forms (**Fig. 9**). The flavin hydroquinone form can either be uncharged or anionic (ionization at the N(1) position) with a pK of 6.5. Thus, anything that will affect the stability of charges (alteration of pK), and the [H^+] will affect the thermodynamics of electron transfer in the free system. The same effect can be obtained by chemical alteration of the flavin ring by the introduction of appropriate substituents and at various positions. A substantial shift of the redox potential occurs upon binding to the target enzyme.

To document the influence of flavin alteration on the two-electron redox potential, a list of 35 analogues and their respective $E_{m,7}$ values taken from the available literature are given in **Table 1**. These values range from a decrease (relative to FAD) of –30 mV to an increase of +230 mV or an overall range of 360 mV. The range for Ox/SQ and SQ/HQ potentials for those analogues listed in **Table 1** are not well documented, although the OX/SQ potential of the 5-deaza-5-carba-FAD has been determined to be extremely low (–700 mV ***(38)***, which would remove this analogue from consideration of one-electron redox transfers due to the unfavorable energetics involved (*see* above). The alterations in potential can be predicted by the inductive influence of the substitu-

Table 1
Oxidation-Reduction Potentials of Flavin Analogues

Flavin analogue	Potential[a] ($E_{m,7}$) (mV)	Reference
1. FAD	–208	*(40)*
Pyrimidine ring-substituted analogues		
2. 4-ThioFAD	–55	*(41)*
3. 2-ThioFAD	–126	*(11)*
4. 3-Deaza-3-carba-FAD	–240	*(1)*
5. 1-Deaza-1-carba-FAD	–280	*(43)*
Pyrazine ring-substituted analogues		
6. 5-Deaza-5-carba-FAD	–310	*(1)*
7. 5-Deaza-5-thia-lumiflavin	+380 (SQ/HQ couple)	*(44)*
Benzenoid ring-substituted analogues		
8. 7,8-a,a,a-Hexafluororiboflavin	+20	*(45)*
9. 8-Fluoro-8-norFAD	–167	*(35)*
10. 8-Bromo-8-norFMN	–148	*(35)*
11. 7,8-Dichloro-7,8-norFAD	–126	*(46)*
12. 7-Chloro-7,8-norFAD	–128	*(46)*
13. 8-Chloro-8-norFAD	–152	*(47)*
14. 7-Bromo-7-norFAD	–154	*(48)*
15. 8-Mercapto-8-norFAD	–290	*(16)*
16. 8-Thiomethyl-8-norFAD	–204	*(35,49)*
17. 8-Methylthiosulfonyl-8-norFMN	–50	*(35)*
18. 8-Methylthiosufinyl-8-norFMN	–161	*(35)*
19. 8-Hydoxy-8-norFAD	–340	*(50)*
20. 8-Amino-8-norFAD	–330	*(35)*
21. 8-Dimethylamino-8-norFAD	–254	*(36)*
22. 8-Methoxy-8-norFMN	–260	*(35)*
23. 8-Ethoxy-8-norFMN	–246	*(35)*
24. 8-H-8-norFMN	–180	*(35)*
25. 6-Methyl-8-norFMN	–200	*(35)*
26. 6-Methylriboflavin	–219	*(51)*
27. 6-HydroxyFAD	–255	*(50)*
28. 6-ThiocyanatoFAD	Not known	
29. 6-AminoFAD	–297	*(50)*
30. 6-MercaptoFAD	Not known	
31. 9-Aza-riboflavin	–135	*(51)*
32. 8-Cyano-8-norriboflavin	–50	*(52)*
33. 8-Formyl-8-norriboflavin	–90[b]	*(53)*
34. 8-Carboxy-8-norriboflavin	–165	*(54)*
35. 8a-Hydroxyriboflavin	–170	*(54)*

(continued)

Table 1 ***(continued)***

[a]All potentials listed are for the two-electron couple of the unbound flavin analogue vs the normal Hydrogen electrode unless otherwise stated.

[b]The listed potential is for the form in which the 8-formyl group is uncomplexed. Intramolecular hemiacetal formation with the riboflavin side chain hydroxyl group lowers the potential to −159 mV *(53)*.

ent on the lowest unoccupied molecular orbital (LUMO) of the oxidized flavin or on the highest occupied molecular orbital (HOMO) of the reduced flavin. A linear dependence of LUMO energy with oxidation-reduction potential has been well-documented with several other classes of redox-active compounds *(39)*. Alterations of these energies and the distribution of electron densities about the flavin ring can provide important insights into the reaction mechanism catalyzed.

Although modifications at just about all positions of the flavin ring will affect the redox potential, substitutions in the pyrimidine moiety will also drastically affect the chemistry of the flavin, and the interaction with the protein. Their use has thus been limited by these considerations. Because there are no documented specific interactions (H-bonding or charge interactions) of the benzene moiety of the flavin with the protein environment, its modification is less likely to alter or result in a protein-induced effect. Consequently, chemical substitutions at positions 6–8 have proven to cause less interference with flavin-protein interactions. In view of these considerations, redox-based studies of flavins modified at these positions (which are also chemically more accessible) have been favored. In order to convey an understanding of the role of electron donating-withdrawing groups in the 7 and 8 positions, which are considered to be respective *meta* and *para* positions to the N(5)-C(4a) reactive center of the flavin ring, we have carried out a correlation analysis of two-electron oxidation-reduction potential with the sum of Hammett σ values for substituents in these two positions. The oxidation-reduction potentials are taken from 20 of the analogues listed in **Table 1** and the values for σ (*para*) (8-substituent) and σ (*meta*) (7 position) are taken from *(55)*. Analogues with substituents in the pyrimidine ring and in the 6 and 9 positions of the benzene ring were not considered since the electronic effects of substituents, as in these positions are difficult to evaluate and also would exhibit steric contributions. An excellent correlation is observed which is described by the equation:

$$E_{m,7} = 182.7\ (\sigma_p+\sigma_m) - 185.7\ (R^2 = 0.94) \qquad (1)$$

This correlation does not include the 8-thio-8-nor-flavin, as the σ value for the anionic thiol substituent is not well characterized. We find that an average of the σ values for SH and S^- provides a reasonable fit which suggests the

possibility that the p*K* of the 8 thiol group is altered from its value of 3.8 in the oxidized form to a higher value in the reduced form. No information in the literature is available on the p*K* of the 8-SH in the reduced flavin analogue. The 8-CH_3SO-analogue was also omitted since the potential may be somewhat altered by the slow reduction of the sulfoxide to the thioether during the redox potential measurement. Similar approaches have been suggested in *(56)* and in the course of writing this manuscript, we became aware of a similar study of the effect of 7 and 8 substitutions on the redox properties of lumiflavin analogues as measured by cyclic voltammetry *(57)*. The results given in *(57)* are similar to the parameters given in **Eq. 1**.

The coefficient (or ρ value) in Eq. 1 is the sum of ρ_p and ρ_m and assumes they contribute equally, which is not necessarily true for a molecular system with the complexity of the isoalloxazine ring. The relative contributions of the sigma values for the 7 and 8 position substituents to the flavin redox potential can be separately evaluated by a two-component multiple regression statistical analysis in accord with the following equation and using the values listed in **Table 2**:

$$E_{m,7}(X_p,Y_m) = \rho_p \sigma_p + \rho_m \sigma_m + E_{m,7}(H,H) \qquad (2)$$

The **results** for this analysis are given below:

$$\rho_p = 203 \pm 10 \text{ mV}/\sigma_p$$
$$\rho_m = 133 \pm 21 \text{ mV}/\sigma_m$$
$$E_{m,7}(H,H) = -178 \pm 4 \text{ mV}$$

The statistical values for this correlation are: $F_{2,19} = 242$ ($P<0.001$), $R^2 = 0.97$

The analysis provides a more valid determination of ρ_p than of ρ_m, as there are more examples of 8- than of 7-substitutions for the flavin analogues listed in **Table 2**. The results given above also demonstrate that the electronic contribution of the 7 substituent is one-third less than that of the 8 substituent. This analysis is of value for its ability to predict which substituents in the 7 and/or 8 positions of the flavin ring would provide a flavin analogue with the desired potential. To illustrate this point, the calculated redox potentials of the 20 flavin analogues listed in **Table 2** were estimated using **Eq. 2** and the parameters listed below it. On substitution of the appropriate σ values for the *para* and *meta* substituents, the redox potentials are readily calculated. Comparison of the calculated and experimental potentials are given in **Table 2** and shown in a graphical form in **Fig. 9**. A linear relationship exists with an expected slope of 1, an intercept (at 0 potential) of zero, and an R^2 value of 0.99. Thus, using **Eq. 2** and the given parameters, it is possible to estimate the potential of any 7 and/or 8 substituted flavin analogue with a high degree of accuracy.

In practice, however, the influence of the protein environment considerably modulates the one- and two-electron potentials of the bound flavin and may

Table 2
Compilation of Sigma Values and Experimental and Calculated Potentials of 7,8-Substituted-Flavin Analogues

Flavin Substituent[a]		Sigma Values		Potentials		
8	7	*para*	*meta*	(mV)[b] (expl)	(mV)[c] (calc)	No.[d]
CH_3	CH_3	–0.170	–0.069	–208	–225	1
CF_3	CF_3	0.54	0.43	+20	–12	8
F	CH_3	0.062	–0.069	–167	–178	9
Br	CH_3	0.232	–0.069	–148	–144	10
Cl	Cl	0.227	0.373	–126	–83	11
H	Cl	0	0.373	–128	–129	12
Cl	CH_3	0.227	–0.069	–152	–145	13
CH_3	Br	–0.17	0.391	–154	–161	14
S^-	CH_3	–1.21	–0.069	–290	—	15
CH_3S	CH_3	0	–0.069	–204	–191	16
CH_3SO_2	CH_3	0.720	–.069	–50	–45	17
CH_3SO	CH_3	0.49	–0.069	–161	—	18
O^-	CH_3	–0.81	–0.069	–340	–354	19
NH_2	CH_3	–0.660	–0.069	–330	–324	20
Me_2N	CH_3	–0.32	–0.069	–254	–255	21
MeO	CH_3	–0.268	–0.069	–260	–245	22
EtO	CH_3	–0.240	–0.069	–246	–239	23
H	CH_3	0	–0.069	–180	–191	24
CN	CH_3	0.660	–0.069	–50	–57	32
CHO	CH_3	0.47	–0.069	–90	–96	33
COO^-	CH_3	0	–0.069	–165	–191	34
CH_2OH	CH3	0	–0.068	–170	–191	35

[a]Sigma values were taken from tables in Hansch and Leo *(55)*.
[b]The experimental $E_{m,7}$ values for each flavin analogue are taken from **Table 1**.
[c]The calculated $E_{m,7}$ values for each flavin analogue are from **Eq. 2**.
[d]The given numbers for each substituted flavin analogue correspond to those in **Table 1**.

differentially alter those of the bound flavin analogue relative to that of the normal flavin due to influences on the expected differential pK values of the redox (SQ and/or HQ) forms of the analogue. This perturbation thus provides additional information on the environment of the flavin binding site. In spite of these additional considerations which makes direct correlations of enzyme rates and other catalytic parameters with the redox potential of the free flavin analogue a dubious exercise, there are examples in the literature where successful correlations have been made including: bacterial luciferase *(35)*, xanthine oxi-

dase *(58)*, and pyridine nucleotide-dependent flavin reductases *(51)*. The number of potential systems that could be studied using flavin analogues with a range of potentials is considerable. A rigorous application to an enzyme system requires not only the influence of the flavin analogue on reaction rates but also requires determination of the potential of the bound analogue under experimental conditions as done by Massey and colleagues to correlate the spectral properties of the charge transfer complex of the FMN of Old Yellow Enzyme with *p*-hydroxybenzaldehyde with the redox potential of the bound flavin analogue *(59)* and to probe the reactivity of flavin-4a-peroxy intermediates in *p*-hydroxybenzoate hydroxylase *(36)*.

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