REVIEW LETTER

CHEMISTRY AND MOLECULAR BIOLOGY OF FLAVINS AND FLAVOPROTEINS

P.HEMMERICH, G.NAGELSCHNEIDER
Fachbereich Biologie, Universität Konstanz, Germany
C.VEEGER
Biochemistry Department, Agricultural University, Wageningen, The Netherlands

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1. Introduction

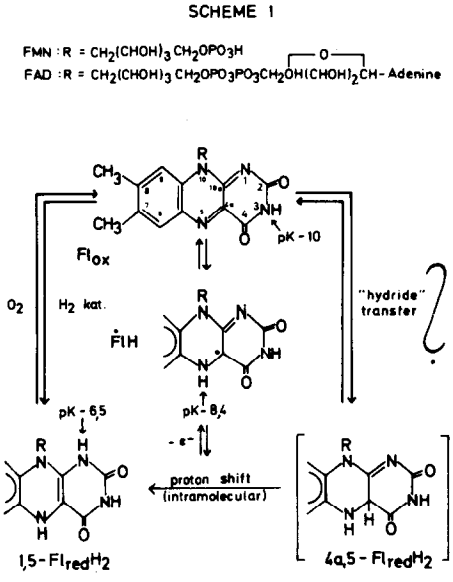
Despite the ubiquity of flavoproteins in nature, the working mechanisms of flavins are not well understood at a "molecular" level. In fact, flavocoenzymes are largely neglected as chemical entities, while molecular physics of flavin and enzymology of flavin, both lively fields of research, still give the impression of being rather divergent disciplines. The situation is improving nowadays, but it is still hard to find chemists interested in flavin problems - quite in contrast to the situation with other coenzymes - and the progress in flavin *chemistry* which has been made since the days of Kuhn and Michaelis is nearly exclusively due to a few biochemists, in particular H.Beinert and V.Massey.

The present review tries to fill a gap by emphasizing the chemical aspects of flavin (cf. scheme 1) though the physical, chemical, and biological aspects of this molecule are equally fascinating and incomprehensible*.

The main complications arise from the great variety of functions that can be assumed by flavins bound to "suitable" apoenzymes, which are summarized in table 1.

Consequently, flavin enzymology tends to be more and more "comparative". Each single study deals with at least some, if not a large variety of different flavoproteins, in attempts to relate function and chemical structure. A detailed account of this is found in the Proceedings of the 3rd International Symposium on Flavins and Flavoproteins held at Durham, N.C., USA, in October 1969 [39], which will appear in print in the near future. In the following, we shall review the highlights of this conference by attempting to present a catalogue of "molecular" problems typical of flavins rather than by describing single enzymes and their experimental characteristics.

SCHEME 1



2. Position of flavin in the electron transfer chains

There are two types of electron chains sufficiently well explored to allow an evaluation of the flavin part as compared to the rest of the chain. In the respiratory system, flavin (rather than ubiquinone or cytochrome *b*) may be the collecting point for electrons from dif-

* See note added in proof.

Table 1
Survey of flavoprotein functions.

Enzyme (Source)	Flavins per mole protein[h]	Other components[d]	Input red./ox.	Output ox./red. other than O_2	Reactions: O_2 + Fl_{red}	Reactions: SO_3^{2-} + Fl_{ox}	Reactions: $1e^-$-acceptors + Fl_{red}	Type of radicals	Ref.
1. *C–H Dehydrogenases*									
Old yellow enzyme (Yeast)	2(FMN)		NADPH/NADP	hFe[a]	+	–	–	red	[1]
D-Amino acid ox. (Kidney)	2(FAD)		$-CH(NH_2)COOH/-COCOOH + NH_3$		+	+	+	red(blue with benzoate)	[1]
L-Amino acid ox. (Snake venom)	2(FAD)		$-CH(NH_2)COOH/-COCOOH + NH_3$		+	+	+	red	[1]
$N(CH_3)$-L-Amino acid ox. (Kidney)	?(FAD)		$-CH(NH(CH_3))COOH/-CH(NH_2)COOH + HCHO$	Fl[b]	+		+		[2]
Monoamine ox. (Kidney)	?(FAD[c])	Cu?	$-CH_2NH_2/-CHO + NH_3$		+				[3]
Pyridoxamine phosphate ox. (Liver)	?(FMN)		$-CH_2NH_2/-CHO + NH_3$						[4]
Pyridoxine-4-ox. (*Pseudomonas*)	?(FAD)		$-CH_2NH_2/-CHO + NH_3$		+		+		[5]
Pyridoxine-5-deh. (*Pseudomonas*)	?(FMN)		Pyridoxine/Isopyridoxal		+		+		[5]
Glucose ox. (*Aspergillus*)	2(FAD)		βD-Glucose/βD-Gluconolactone		+	+	–	blue pH < 9 red pH > 9	[1]
Glycollate ox. (Spinach)	2(FMN)		$HOCH_2COOH/OHCCOOH$		+	+	–	red	[1]
Pyruvate ox. (*L.delbrückii*)	?(FAD)	TPP[f]	$CH_3COCOOH + P_i/CH_3CO\text{-}P + CO_2$		+		+		[6]
Pyruvate deh. (*E. coli* B)	4(FAD)	TPP[f]	$CH_3COCOOH/CH_3COOH + CO_2$	hFe[a]			+		[7]

Enzyme (Source)	Flavins per mole protein[h]	Other components[d]	Input red./ox.	Output ox./red. other than O_2	Reactions: O_2 + Fl_{red}	Reactions: SO_3^{2-} Fl_{ox}	Reactions: $1e^-$-acceptors + Fl_{red}	Type of radicals	Ref.
Oxalate ox. (Mosses)	?(FMN or Ribofl.)		HOOCCOOH/2 CO_2		+				[8]
Acyl-CoA deh. (Liver)	2 ?(FAD)		C_6-C_{16}acyl-CoA/ 2,3 dehydro acylCoA	Fl[b]	–	–	–	blue	[1]
NAD-peroxidase (*Streptococcus*)	?(FAD)		NADH/NAD	H_2O_2/H_2O	+		+		[9]
NADH-cyt.b_5 red. (Liver micros.)	1(FAD)		NADH/NAD	hFe[a]	+		+	blue	[1]
NADPH-cyt.*c* red. (Liver micros.)	2?(FAD)		NADPH/NADP	hFe[a]	+		+	blue	[1]
NADH-deh. (Heart mito.)	1(FMN)		NADH/NAD	hFe[a] SFe[d], CoQ			+	red	[10, 11]
NADPH-quinone ox.red. (Hog liver)	?		NADPH/NADP or NADH/NAD	quinones	–?		–?		[12]
NADPH-vit.K ox.red. (Liver)	1(FAD)		NADPH/NADP	naphtoquinones	–?		–?		[13]
L-6-OH nicotin ox. (*Arthrobacter*)	2(FAD)		>CH–N</>C=O + HN<		+		+	red	[1, 14]
Salicylate oxyg. (*Pseudomonas*)	1(FAD)		salicylate/catechol NADPH/NADP		+		+	red(blue with substrate)	[1, 15]
p-OH-benzoate oxyg. (*Pseudomonas*)	1(FAD)		*p*-OH-benzoate/3,4 di-OH-benzoate NADPH/NADP		+	–			[1, 16]
Xanthine ox. (Milk)	2(FAD)	Mo,SFe	xanthine/uric acid		+		+	blue	[17–19]
Aldehyde ox. (Liver)	?(FAD)	Mo,SFe	–CHO/–COOH		+		+		[19]
Succinate deh. (Heart mito.)	1?(FAD[c])	SFe	$HOOCCH_2CH_2COOH$/ HOOCCH=CHCOOH	Fl[b], CoQ	–		+		[20, 21]

Enzyme (Source)	Flavins per mole protein[h]	Other components[d]	Input red./ox.	Output ox./red. other than O_2	Reactions: O_2 + Fl_{red}	Reactions: SO_3^{2-} Fl_{ox}	Reactions: 1e⁻-acceptors + Fl_{red}	Type of radicals	Ref.
Lactate deh. (Yeast)	4(FAD)		$CH_3CHOHCOOH/CH_3COCOOH$	hFe[a]			+		[22]
Lactate ox. (Myobact.)	(FMN)	Zn^{2+}, TPP[f]	$CH_3CHOHCOOH/CH_3COOH$ CO_2	hFe[a]	+	+	+	red	[15, 1]
Glycerol phosphate dehydr. (Mito.)	1(FAD)	SFe	$CH_2OHCHOHCH_2O\text{-}P/$ $OHCCHOHCH_2O\text{-}P$	SFe[d], CoQ? Fl[b]?			+?		[23]
Dihydrorotate deh. (Zymobact.)	1(FMN) + 1(FAD)	SFe	dihydro orotic acid/ orotic acid	NAD/NADH	+				[19]
Choline deh. (Liver mito.)	1(FAD)	SFe	$(CH_3)_3\overset{+}{N}CH_2CH_2OH/$ $(CH_3)_3\overset{+}{N}CH_2CHO$	NAD/NADH			+		[24]
2. *–S–H– Dehydrogenases*									
Dihydrolipoamide deh. (Mito.)	2(FAD)	S_{act}[e]	dihydrolipoamide/ lipoamide	Fl[b]?	–	–	+	none[g]	[1,25, 26]
Glutathione red. (Yeast)	2 (FAD)	S_{act}[e]	–S–S–/–SH		–	–	+	none[g]	[1, 25, 26]
Thioredoxin red. (*E. coli*)	1(FAD)	S_{act}[e]	–S–S–/–SH		–	+	+	blue	[1,25 26,27]
3. *e⁻-Transferases*									
Ferredoxin-NADP red. (Spinach)	1(FAD)		SFe^{II}/SFe^{III}	NADP/ NADPH, hFe[a]	+	–	+	blue	[1, 27–29]
Electron Transferring Flavoprot.	1(FAD)		Fl[b]	Fl[b]	–		+	red	[1]
Flavodoxin (*Peptostreptococcus*)	1(FMN)			chloroplasts	+	–	+	blue	[1, 30]
Azotobacter flavoprotein	?(FAD)		?	?	+	–	+	blue	[1]
Phytoflavin (Alga)	1(FMN)		NADP/NADPH	chloroplasts	+		+	blue	[31]

Enzyme (Source)	Flavins per mole protein[h]	Other components[d]	Input red./ox.	Output ox./red. other than O_2	Reactions: O_2 + Fl_{red}	Reactions: SO_3^{2-} + Fl_{ox}	Reactions: $1e^-$-acceptors + Fl_{red}	Type of radical	Ref.
4. *Other redoxactive flavoproteins*									
Sulfite red. (*Salmonella*)	2(FAD + FMN)		SO_3^{2-}/S^{2-}	SFe[d], hFe[a]			+	blue	[32]
Sulfate red. (Bact.)	1(FAD)	SFe	$AMP-SO_3H/AMP + SO_3^{2-}$	SFe[d]	+?	+			[33]
Nitrate red. (Bact.)	?(FAD)	Mo	NO_3^-/NO_2^- NADPH/NADP	hFe[a]			+		[34]
5. *Redox-inactive flavoproteins*									
Glyoxylate carboligase (*E. coli*)	?(FAD)	TPP[f]	OHCCOOH/OHCCHOHCOOH + CO_2		–	–	+?		[1, 35]
Oxynitrilase (Almonds)	1(FAD)		$C_6H_5CHOHCN/C_6H_5CHO$ + HCN		+	+		red	[1]

[a] hFe = heme-iron, $Fe^{II} \rightleftharpoons Fe^{III}$

[b] Fl = Flavin (It is assumed, that natural electron transport runs from initial dehydrogenases in many cases through other flavoproteins, like "electron transferring flavoprotein" [1]; before it reaches ubiquinone or cytochromes.)

[c] "Covalently bound flavocoenzyme", cf. [36].

[d] SFe = iron-sulfur protein (In many cases, cf. "other components", this table, SFe is an integral part of the flavoprotein)

[e] S_{act} = reactive disulfide center at the enzyme active site, yielding a diamagnetic FlS-intermediate (cf. p. 80), which prevents formation of radicals in the case of the "2 FAD/Mole-Enzymes".

[f] TPP = thiamine pyrophosphate (It seems at least possible, that the immediate substrate of flavin in the TPP-flavoproteins is the "carbanion" [37] (or enamine) of the "active aldehyde".

[g] In the presence of NAD excess the red diamagnetic intermediate can be photolysed to yield ~50% $Fl^{\cdot}$ presumably of the blue type [38].

[h] The numbers in this column are subject to potential error in so far as they indicate in many cases the smallest *active* subunit, but in some cases it is not established, whether the observed subunit is active as monomer. Therefore the given numbers may in fact be greater by factors of 2 or 4 and in particular "one-flavin-enzymes" may act as dimers [71]. This is supported by the fact, that such proteins, e.g. phytoflavin [31], interact rapidly with themselves, whereas "two-flavin-enzymes" in general do not.

Empty space in a given column means, that no data are available.

This table is an extension of earlier ones given by Massey and Palmer [1; 81].

ferent substrates, from which the (more linear?) transport through the cytochromes originates [40, 41]. Flavins may be rate-limiting in mitochondrial respiration, since all other chain components are present in excess (nicotinamides: flavins: ubiquinone: cytochrome $a \approx 100:1:100:10$ [40]). In photosynthesis, flavin pilots cyclic and non-cyclic electron flow [28, 42] and energy conservation. The proposal has even been made that flavin of ferredoxin-NADPH-oxidoreductase is the primary electron acceptor in chlorophyll *P* 700 complex [43]. Though this result of a model experiment should be taken with great caution, (as should any positive conclusions from model experiments on a biological mechanism), this seems to be an interesting and, at least, physicochemically sound idea. In both microsomal and (adrenal) mitochondrial oxygenation, flavin seems to control not only cytochrome reduction but also, independently, O_2-activation [44, 45]: till now, this has been ascribed to direct interaction of NADPH and cytochrome *P*-450, but since this would be the first example of a flavin-independent transfer of redox-equivalents from a reduced nicotinamide to O_2, the directness of NADP → *P*-450 interaction remains doubtful.

From this two other problems arise, the problem of "interflavin contact" in proteins and the problem of "one-electron versus two-electron transfer". Progress in this context comes from the work of Weatherby and Carr [46], who demonstrated 1,2-dihydrophthalic esters to be dehydrogenated via their carbanions by flavoquinones *in the dark*. Hence there is increasing support for the idea that flavin-dependent dehydrogenation means carbanion oxidation (cf. section 5, below).

3. "Electron pair splitting" and "interflavin contact" in flavoproteins

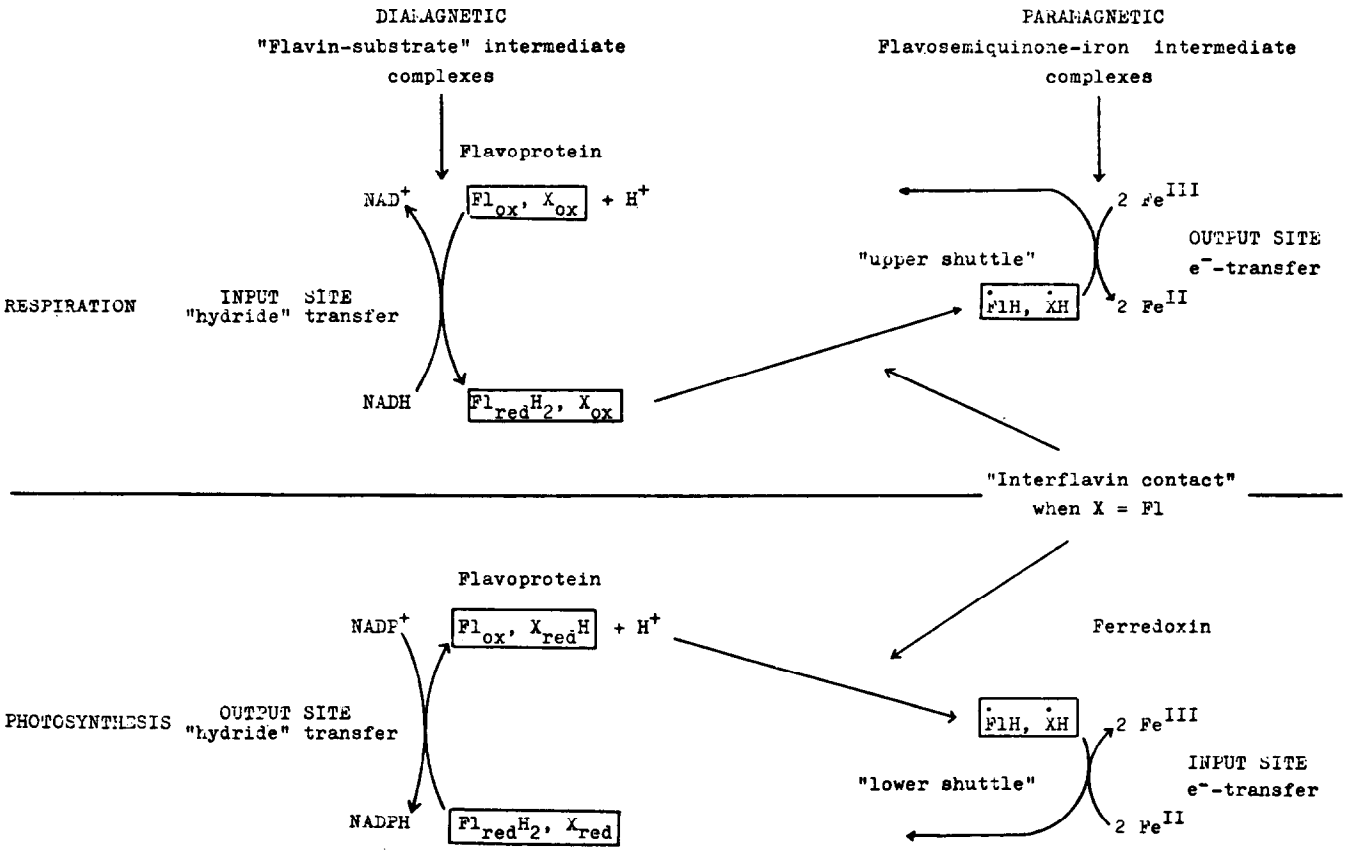
When redox equivalents are transferred through flavin from, for example, NADH to cytochrome or ubiquinone, flavin accepts two electrons at a time together with a proton, i.e. a "hydride equivalent". As polyphonic as the flavoprotein concerto might be in the literature, no chemical argument has been raised, as far as we can see, to support H-radical transfer from NADH to flavin. Since on the other hand, the acceptor site, i.e. hemoprotein or iron-sulfur protein (cf. footnote*), will certainly not accept more than one electron at a time, Singer's [47] early postulate that flavin "splits" the electron pair has held its ground. The situation is less clear with sulfur-containing flavin substrates, but there is increasing support for the assumption that sulfate [33] and sulfite [32] reduction by flavoproteins, and also (artificial) dithionite [31] and mercaptide [25] oxidation, are $2e^-$-transfers. The same question applied to flavin-dependent O_2-activation is also entirely open (see below).

"Two-electron transfer" requires flavin to shuttle between Fl_{ox} ("fully oxidized" state or "flavoquinone") and $Fl_{red}H^-$ ("fully reduced" state or flavohydroquinone" (cf. scheme 1)). "One-electron transfer", on the other hand, requires stabilization of the intermediate radical state or "flavosemiquinone" (see below). Flavin may then shuttle between flavoquinone and flavosemiquinone, on the one hand, or flavosemiquinone and flavohydroquinone, on the other hand. Both levels represent a drastic difference in potential: for the first time, the potentials of the upper *and* the lower one-electron level have been determined in a flavoprotein [48]: they differ as much as 260 mV from each other, and the lower one (−370 mV) is nearly as low as the hydrogen electrode under physiological conditions (−420 mV).

If a flavoprotein "splits" electron pairs, then it may be postulated that the radical electrons can be evolved at one and the same potential, since e.g. cytochrome would not accept electrons of such different energy levels at a comparable rate. But how could this be done? A solution to this problem is outlined in scheme 2.

In order to evolve a second radical electron at the same potential, an additional redox-active group *X* would be required which must "comproportionate" (respiration) or "disproportionate" (photosynthesis) the system to yield the second redox equivalent at the required potential. Though nature might also have other groups *X*, the easiest way out of the dilemma is for *X* to be a second flavin, i.e. "interflavin contact". In fact, the number of flavoproteins which contain two or more moles of flavin per mole of protein, is quite high. All these might undergo *intra*molecular interflavin contact, which is very difficult to prove

* "Iron-sulfur-protein" is a nomenclature recently introduced instead of "redoxactive non-heme iron protein".



Scheme 2. Flavin-dependent electron pair "splitting" and possible equilibration of 1e⁻-potentials.

experimentally. Simple "one-flavin enzymes", i.e. ones which do not contain any other redox active group, on the other hand, seem to react rather fast even intermolecularly [31, 29]. This problem needs further attention (cf. also footnote h in table 1).

The steric requirements of such an interflavin contact are not necessarily those of a sandwich complex, since a π-complex (as found in the protein-free flavin system = "flavoquinhydrone") would require large conformational changes, if involved in flavoprotein catalysis. Accordingly a flavoquinhydrone charge-transfer absorption is not known in flavoproteins. A one-dimensional contact (σ-interaction) should be sufficient to explain electron exchange between two flavin molecules. A statement can be made on the basis of what is known of the submolecular structure of flavosemiquinone from the studies of Ehrenberg and his group [49–51]: an "interflavin linkage" HFl–FlH of σ-character must involve a connecting point of high spin density in the radical $\dot{F}$lH.

Since the "pyrimidine subnucleus" of the flavosemiquinone molecule (positions 1–4) (cf. scheme 1) exhibits very low if any spin density, a covalent linkage HFl-FlH can only involve N(5) in a hydrazine-like structure with a dihedral angle of 90° between the flavin halves. Such a linkage should be kinetically, as well as thermodynamically, rather unstable. Hence, it could only be stabilized within a macromolecular frame, while the protein-free system would prefer the π-complex. Supposing, however, that an interflavin N(5)-linkage existed in a half-reduced "two-flavins-per-mole" flavoprotein, its spectral properties should be

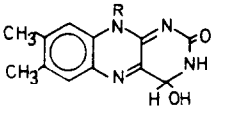
nearly equal to those of the fully reduced $Fl_{red}H_2$. This possible source of error should be kept in mind.

4. Functional sites of the flavin molecule

Like most cofactors in biochemistry, flavin is usually represented by a "three-letter" word, i.e. FAD or FMN. But certainly electrons cannot be considered to flip to and from this biocatalyst like little sparks. If we go a little further into the molecular details of this catalysis, we can refer to the overlap between flavin and substrate π-orbitals within the catalytically active complex. This may be true for some substrates, but no theoretical chemist could ever explain "π-overlap" between a heteroaromatic donor-acceptor molecule and, e.g. substrates like glycine, glucose, and fatty acid thio esters. On the other hand, we might assume that there is little or no contribution from the flavin to the energy of the reactive complex, i.e. we might postulate that the apoprotein does everything and the flavin merely behaves as an electron sink. This picture would not represent - in our opinion - anything but a lag of chemical thinking in biology. If we continue along this line, we end up at the same good old vitalism which believes in a certain "bios" mysteriously inherent in proteins, which our ancestors believed to be inherent in whole cells. We assume, therefore, that the question why nature uses flavin and not just any other quinoid oxidoreductant of suitable potential, cannot "simply" be answered by allowing flavin, and nothing else, to fit in the cofactor "hole" of the apoenzyme.

In the meantime, this latter assumption could be disproved in some cases. There is a steady increase in flavo-apoenzymes available for reconstitution experiments with flavocoenzyme analogs. The results from the McCormick group at Cornell [52–58] show that the specificity for flavin binding is not as high as one might expect. Flavin analogs may in certain flavoproteins replace the natural coenzymes forming stable *and* in some cases even active holoenzymes. Veeger's group [59, 60] showed for lipoamide dehydrogenase and glutathione reductase, that if both enzymes are reconstituted from apoenzyme and FAD, the activity returns, but the reconstituted enzymes are inhibited by FMN, in contrast to the holoenzymes as isolated. This suggests, that a variety of protein conformations may support the activity. These studies deserve more encouragement.

From the data available at present, it emerges that "π-overlap" can hardly be the mode of the flavin-substrate contact, since for efficient π-overlap a rather large area of contact is needed, and this requires less steric restrictions than obviously exist. Hence, σ-character must be expected for flavin-substrate contact, more definitely than in the case of interflavin contact. This, again, requires a specific "active site at the flavin molecule" for substrate interaction. At the same time, this justifies "model studies" of flavin structure and reactivity in protein-free, i.e. "chemical" systems. The two acceptor sites, C(4a) and N(5), as revealed by unpaired spin density evaluation of the radical species [49–51], have been confirmed by the elegant work of Massey et al. [61, 62], in which they showed that amino acid oxidases reduced by BH_4^- in position 4 (cf. I) are still reactive enzymatically.



I

Redox activity through C(4a) clearly involves an "out-of-plane" attack by substrate, while N(5) allows an "in-plane" approach. C(4a) attack, therefore, involves by itself a stereochemical problem, since approach from above and below yields enantiomeric flavin-substrate or flavin hydride complexes. A subsequent proton shift from C(4a) to N(1) may increase the asymmetry (cf. scheme 1*). The problem to be tackled is the velocity of this proton shift as related to the velocity of hydride attack at C(4a). N(5)-attack, however, is stereochemically also not indifferent, if 1,5-dihydroflavin is non-coplanar (see below). This opens two new problems inherent in flavin biocatalysis: What entity is actually transferred in the "two-electron" transfer and, consequently, what is the stereochemistry of this transfer?

* H.Gawron was first to propose such a structure [95].

5. "Hydride" versus group transfer

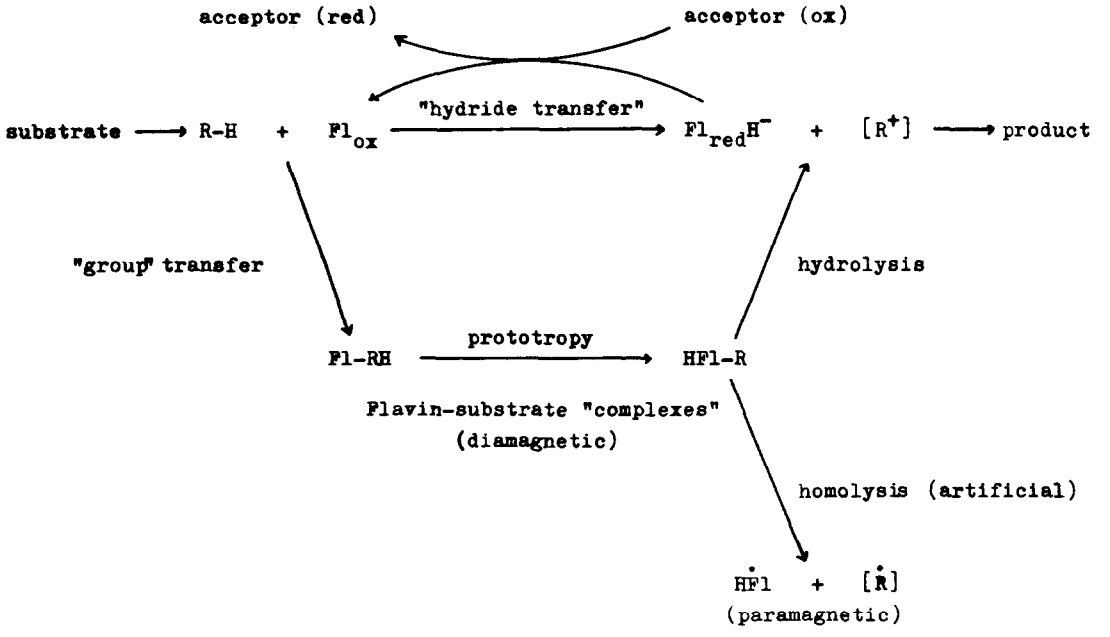
A chemical species R-H may be dehydrogenated to yield (in some "complexed state" defined by secondary reactions) $\dot{R} + \dot{H}$, $R^+ + H^-$, or $R^- + H^+$ depending on the state of polarization of the bond R–H at the time of the bond rupture (charge control) and also depending on the preference of the acceptor sites (frontier orbital control [63]). Homolysis of R–H can be excluded as the operative mechanism (cf. above 2). In general, breakage is thought to yield R^+ and H^-, though this introduces several new complications which are normally not taken into account: First, direct generation of R^+ would, for product formation, require a second step of activation which ought to be independent of flavin. This step must be faster, or at least synchronous, with the rate-determining step of R–H rupture, if internal transfer of R^+ to the protein is to be avoided, which would lead to protein alkylation. Such an alkylation mechanism has been assumed by Hellerman and Coffey [64] who, in the case of D-aminoacid oxidase ($R^{\oplus} = R'C^{\oplus}(NH_2)COOH$), "trapped" R^+ at a lysine site of the apoenzyme. But this reaction has been shown to be unessential by Massey et al. [61], since the alkylated protein happened to have full enzymatic activity.

Second, hydride transfer is a chemically well established reaction, e.g. in metallohydride reduction of carbonyl compounds, and quinoid systems are known to react rather slowly with true hydride equivalents. Furthermore, true hydride transfer requires a transition state equivalent to the three-center bond of hydroboranes and not equivalent to a hydrogen bridge. Given an apolar bond R–H, it is less difficult to imagine a polarization $R \leftarrow H$ by the apoprotein with subsequent deprotonation and reaction of flavin with R^-, than it is to visualize the reverse case of a polarization $R \rightarrow H$ with subsequent hydride acceptance by the flavin. This is, in contrast to the situation encountered with nicotinamides which presumably undergo true hydride transfer from alcohols, favored by the positive charge of $NAD(P)^+$. These arguments may not be sufficient to render the notion of hydride transfer with flavins obsolete, but they may help to free one's mind for consideration of the opposite case, viz. group transfer, as an equally possible working mechanism of flavin-dependent dehydrogenation. Group transfer has been shown to occur "chemically" from e.g. phenyl acetic acid to yield N(5) - as well as C(4a) - benzylated dihydro flavins [65]. A change in structure as small as that from $C_6H_5CH(CH_3)COOH$ to $C_6H_5CH(OCH_3)COOH$ appears to switch the reaction from group to hydride transfer.

We used the words "it appears" since there is yet another possibility: perhaps group transfer does occur in either case, but the difference in the overall react originates from subsequent solvolysis of the less stable substituent. In fact, group transfer and subsequent hydrolytic cleavage of the intermediate alkyl dihydro flavin $HFl_{red}R$ may simulate hydride transfer (cf. scheme 3). This is all the more possible if $HFl_{red}R$ requires little or no activation, even in the absence of protein, to undergo solvolytic dealkylation. In other words, flavin may dehydrogenate R–H accepting at first R^-, and then releasing R^+ hydrolytically. It is an important finding in this context that, as it emerges from ENDOR-measurements [66], there is a region of "active water" around N(5) in certain flavoproteins (cf. below, "type A").

Supporting evidence comes also from H^+-exchange of succinate dehydrogenase. Zeijlemaker has shown [67], that the addition of succinate or oxaloacetate to the oxidized enzyme gives an absorbancy increase with the maximum at 310 nm (succinate) and 300 nm (oxaloacetate). This would be consistent with an N(5)-alkylation or acylation, respectively [68].

If, however, straightforward hydride transfer proves to be real, there is still biological importance in the recently found group transfer reactions. As shown by de Kok, Veeger and Hemmerich [68] for acyl transfer and by Komai and Massey [69] for alkyl transfer towards flavin, artificial residues can be introduced into flavoproteins as stable "flavin labels" [69, 70]. The changed activities of the so modified enzymes prove to be extremely interesting especially in enzymes with multiple cofactors like xanthine oxidase. Such reactions also may help in understanding why some flavoproteins require FMN and others FAD, since they may allow conclusions to be drawn as to whether there is flavin-adenine contact in FAD enzymes. The biological importance of this contact seems to be entirely unestablished at present. Could indeed the AMP residue of FAD be nothing but a means of filling a cavity in the apoenzyme? At least, there is yet no piece of straight evidence for an intramolecular charge transfer complex of flavin and aden-



Scheme 3. Flavin-substrate complexes and hydride versus group transfer paths in flavin dependent substrate dehydrogenations.

ine in FAD enzymes, but in some cases there is evidence against [68, 71, 72]. More results on this subject may be expected from flavoprotein *photo*chemistry.

6. The potential biological relevance of electronically excited flavins

In fact, all the above mentioned *chemical* group transfer reactions towards flavin are photoinduced, i.e. the active species is the excited flavoquinone triplet $\dot{\dot{F}}l_{ox}$*. If the list of "substrates" undergoing photochemical dehydrogenation by flavin is compared with the list of substrates of flavin-dependent dehydrogenases, strong similarities can be detected: amines (but not acylamines or ammonium ions), mercaptans (but not alcohols) and dihydronicotinamides. Certainly, there are exceptions to this rule: the flavin-dependent glycerol phosphate dehydrogenase is an alcohol dehydrogenating protein [23, 73], while photochemically alcohol dehydrogenation by flavins occurs with a reasonable velocity only in the ribityl side chain [74], viz. intramolecularly. But the question still stands, whether in flavoprotein catalysis, an excited state of flavoquinone could be reached, which has failed to be detected up to now because of its short lifetime?

A second question may be asked along these lines: Is there a flavin-dependent photobiology? Hastings [75] found that flavin is involved in bacterial bioluminescence, and Cormier et al. [76] have recently shown that the hitherto unexplained bacterial bioluminescence spectrum is identical with the fluorescence spectrum of $FMNH^+$ (λ_{max} = 483 nm), not FMN (λ_{max} = 520 nm).

According to Delbrück [77], it seems probable that a specialized flavin undergoes a photochemical reaction *in vivo*, with a high quantum yield, and that this reaction is used throughout the plant kingdom, from bacteria to higher plants, to control a large variety of physiological processes [78]. An interesting chemiluminescent model based on flavin has been developed by Stone, Vorhaben and Steele [79]. Instead of $FMNH^+$, it depends on $FMNCu^+$, which explains the emission maximum at 525 nm compared to the natural 480–490 nm.

A third question would be: Is there a flavin de-

pendent mechanism of energy conservation?

The synthesis of the first "squiggle" ("≈ X" = energy rich bond or membrane state) in the respiratory chain is certainly connected with "NADH-dehydrogenase", but this flavoprotein also contains redox active iron and sulfur [11]. Questions and questions....

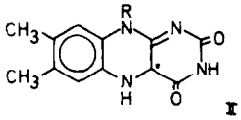
7. The biological importance of flavin radicals

Today, all available evidence, mainly ESR-data (e.g. [80]), agrees that radicals are essential intermediates in flavin biocatalysis only in those cases, where electron transfer occurs towards one electron donor-acceptor systems in the strict sense, i.e. heme-iron and non-heme-iron (ferredoxin). With molybdenum the situation is not clear, since the stoichiometry of Mo-oxidoreduction in molybdo-flavoenzymes is still unsettled [18]. On the other hand, "artificial" radicals are known to be formed in most flavoproteins by means of either i) one-electron oxidoreduction, e.g. $Fl_{red}H^- + Fe(CN)_6^{3-} \rightarrow \dot{F}lH + Fe(CN)_6^{4-}$, ii) comproportionation $Fl_{red}H_2 + Fl_{ox} \rightarrow 2\,\dot{F}lH$, or iii) homolysis of flavin-substrate complexes $Fl{-}X \rightarrow \dot{F}l + \dot{X}$. Case i) is difficult to demonstrate since the $\dot{F}lH$ formed reacts more rapidly (to yield Fl_{ox}) than $Fl_{red}H^-$, due to the negative charge of the latter. Case ii) can be achieved experimentally only in the *inter*molecular mode and this is known to be very slow for many flavoproteins. iii) is the most common way, the nature of X can be manifold: it may be "active sulfur" from a cyst(e)ine residue at the active site [25, 26, 80], it may be "active alkyl" from group transfer (see above scheme 3), it may be "active oxygen" (see below).

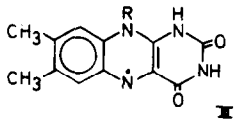
Generalizing the concept of Massey and Palmer [81], one might distinguish four classes of flavoproteins according to their behaviour at half reduction in the absence of substrates:

A) Flavoproteins exhibiting stoichiometric amounts of blue semiquinone,
B) Flavoproteins exhibiting stoichiometric amounts of red semiquinone,
C) Flavoproteins exhibiting no paramagnetic intermediates,
D) Flavoproteins exhibiting non-stoichiometric amounts of semiquinone.

The red radicals of class B have been identified as flavosemiquinone anions $\dot{F}l^-$ [82]. The blue radicals of class A have been identified as neutral semiquinones $\dot{F}lH$ [51]. In fact (cf. table 1), glucose oxidase belongs to class A *and* B, depending on pH. The structure of $H\dot{F}l$ has been established as (II), i.e. 5-$H\dot{F}l$, and not, as assumed earlier, as 1-$H\dot{F}l$ (III).



II



III

This leads to the conclusion that the thermodynamic stabilization of the radical by these apoenzymes

$$Fl_{ox} + Fl_{red}H_2 \underset{\text{free coenzymes}}{\overset{\text{holoenzymes}}{\rightleftharpoons}} 2\,\dot{F}lH \underset{pK = 8.4}{\rightleftharpoons} 2\,\dot{F}l^- + 2\,H^+ \quad [82, 84]$$

is governed by a prosthetic group pointing towards N(5) of the coenzyme to form a strong hydrogen bridge, like $-COO^-$.....$H\dot{F}l$ in the blue radical and $-NH_3^+$·····$\dot{F}l^-$ in the red radical (see fig. 1). This is chemically reasonable, because neither Fl_{ox} nor $Fl_{red}H_2$ allow hydrogen bridging towards N(5), so that the above equilibrium is displaced towards the right in the holoenzyme.

From this it becomes obvious that in the class A and B flavoproteins N(5), i.e. the "active site of the flavin nucleus", is "protected" in the radical state, so that no "in-plane" transfer of electrons can occur. Clearly, these radicals are stable as long as the hydrogen bridge at N(5) is not removed. We propose that there are only two biologically possible ways for the essential "discharge" of a flavin radical, whether by uptake or donation of a single electron:

1) "Interflavin" contact
2) Metal contact.

The interflavin contact is governed by the above equilibrium, which should be fast enough to be catalytically essential. Metal contact, on the other hand, is characteristic, as we postulate, for class D flavoproteins. In fact, a class A or B flavoprotein like NADPH-ferredoxin oxidoreductase may change into class D via change of quaternary structure, i.e. formation of a flavoprotein-ferredoxin "complex" [29, 85], which loosens the hydrogen bridge at N(5). We consider it significant, that in metalloflavoproteins con-

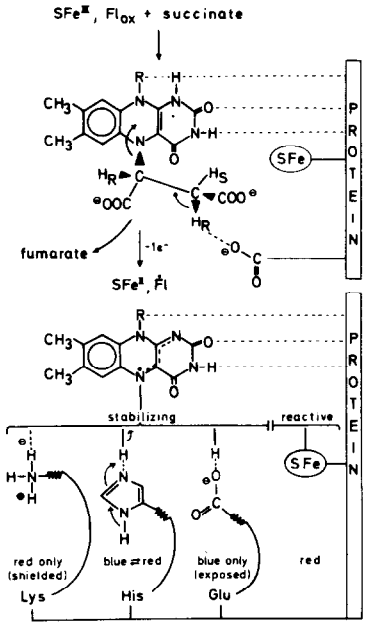


Fig. 1. Flavin-substrate-complex (above), based on the data of Rétey et al. [83] and Dervartanian et al. [21] and stabilised radical (below) [51].

trary to metal-free flavoproteins the radical "yields" at half reduction never exceed 50% of total flavin†. We assume, that the "missing" part of the flavin is complexed via N(5) to the metal. Such flavosemiquinone iron chelates are indetectable by ESR [50] and their optical spectra [86] will not be easily distinguished from those of iron-sulfur complexes.

Class C, finally, contains those flavoproteins which exhibit a cyst(e)ine residue participating in the catalysis like dihydrolipoamide dehydrogenase and glutathione reductase [25–27]. Both internal redox systems, i.e. flavin and disulfide, are in covalent contact at 2e$^-$-reduction of the enzyme:

$$Fl_{ox} + \overset{S}{\underset{S}{\wedge}} \rightleftharpoons^{2e^-,\,2H^+} HFl{-}S \quad \overset{}{\underset{HS}{\wedge}}$$

Radicals can only be generated by denaturation through homolysis of the Fl–S bond or blocking of the sulfur component by thiophilic agents. The point of attachment of sulfur to the flavin has been proposed to be C(4a) [87].

† Experimental fact communicated privately by Dr. H.Beinert who, in contrast to us, would not insist on its significance.

The points in this section have been made neglecting any formation of ternary complexes, though there are many indications that only the full assembly of donor (input substrate), flavin and acceptor (output substrate, cf. table 1) shows full catalytic activity [83]. It may even be that in "two-flavin enzymes" one flavin is needed specifically to "complex" the donor, whereas the other flavin, in contact with the first one, would specifically "complex" the acceptor. But it seems premature to speculate about structures involved.

8. The stereochemistry of fully reduced flavin

Two-electron transfer, whether hydride or group transfer will create asymmetry, since it leads from a planar flavoquinone to a non-planar (cf. scheme 1) dihydroflavin. In fact, not only the new 4a,5 dihydroflavin, but also the "normal" 1,5 dihydroflavin (or "flavohydroquinone") is a bent molecule, in the crystalline state as well as in solution [88]. The dihedral angle of the "butterfly wing" molecule varies between 140–160° in crystalline samples. One must assume, that in the protein bound state,the inversion of the nitrogen centers in position 5 and 10 is impeded, and that the apoprotein prefers one or the other of the enantiomers. This would create a

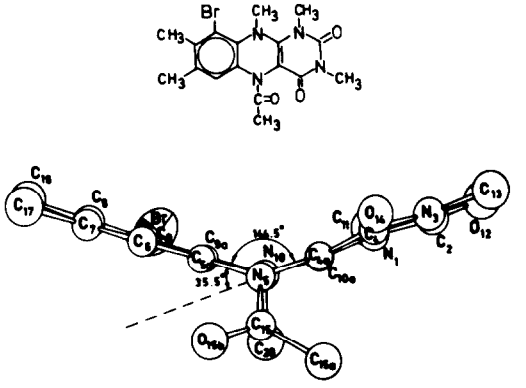


Fig. 2. Three dimensional structure of flavohydroquinone [88, 89]. Courtesy of Dr.Peder Kierkegaard. Replacement of the acetyl group by hydrogen increases the dihedral angle from above 144.5° to 159°. The overcrowding effects from the (N)CH3 and the (C)Br groups are not very significant, since the corresponding flavoquinone species are coplanar within the error of crystallography.

situation similar to that found with nicotinamide dependent dehydrogenases, with the only difference being that the removal of the coenzyme from the flavoprotein brings about immediate racemisation. Recent results on the stereochemistry of flavin-dependent succinate dehydrogenation [83], as well as on the differences between functionally closely related flavoproteins, as D- and L-amino acid oxidases [68], point to the existence of flavin asymmetry. Likewise, such considerations may be relevant to the question as to why some fully reduced flavoproteins are oxygen activators while others are not.

9. Flavin dependent activation of oxygen

The fact that the highly symmetric triplet molecule O_2 is rapidly attacked by reduced flavin, has been attributed mainly to the reactivity of flavosemiquinone present in minute amounts. Recent studies with flavoprotein radicals, however, show unequivocally that flavohydroquinone is the active flavin state, not semiquinone [31, 51]. In general, blue flavoprotein radicals even appear quite inert towards O_2 (cf. table 1). This provokes the suggestion that flavin radicals are autoxidized "through disproportionation".

$$2\,\dot{H}Fl \rightleftharpoons Fl_{ox} + Fl_{red}H_2 \xrightarrow{O_2} Fl_{ox} + HFlOOH$$

Studies with model flavohydroquinones [90] lead to the conclusion that autoxidation occurs more rapidly with the (vibrationally excited) flat state of $Fl_{red}H_2$ than with the bent ground state (cf. fig. 2). This explanation gains independent support from the well known autocatalysis in the chemical reaction of $Fl_{red}H_2$ with O_2 [91]: since this phenomenon can no longer be explained by radical formation, the "autocatalyst" must be Fl_{ox}. This is easily understood if one takes into consideration that a half reduced free flavin system contains not only radicals, but also "dimers" of the composition $Fl_{red}-Fl_{ox}$, i.e. flavoquinhydrone charge transfer complexes in which the Fl_{red} moiety is flattened by the presence of Fl_{ox}. In the protein bound Fl_{red}, a flat functional group like adenine or tryptophan might serve the same purpose.

Although at present no decisive evidence can be given, particularly regarding molecular structure, there is a wide belief in a "complex" HFlOOH [1] as essential intermediate of flavin autoxidation. Again, this complex has a tendency towards homolysis, yielding flavosemiquinone and superoxide radical $\dot{O}_2^-$ [92–94]. This latter species can only be observed under alkaline conditions, where its lifetime is sufficiently long. Hence, the obvious failure to detect $\dot{O}_2^-$-formation at neutral pH might equally well be due to the instability of this species as to $\dot{O}_2^-$ being an "alkaline artefact" in flavoprotein autoxidation.

The understanding of flavin biocatalysis in terms of molecular structure is at an early stage. The ideas outlined here, though admittedly speculative, should be acknowledged at least as chemically reasonable and therefore be a challenge to discussion, even if a fair portion of them may eventually be disproved in the near future.

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Note added in proof

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