Review Letter

FLAVIN AND 5-DEAZAFLAVIN: A CHEMICAL EVALUATION OF 'MODIFIED' FLAVOPROTEINS WITH RESPECT TO THE MECHANISMS OF REDOX BIOCATALYSIS

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Received 30 August 1977

1. Introduction

Over the last several years, there have been reported a number of studies on flavoenzymes in which the native flavin coenzyme has been replaced by a 5-deazaflavin. These studies and some of the pertinent findings are summarized in table 1. The rationale for these studies is stated in the opening sentence of a recent paper by Hersh and Jorns [1]:

> 'The similarity in chemical properties of deazaflavins as compared to normal flavins has provided the basis for using these analogues to study both nonenzymatic and flavoenzyme reactions'.

It is the purpose of this review letter to emphasize that in fact the chemistry of flavins and deazaflavins is fundamentally different and that the chemistry of deazaflavins resembles much more that of pyridine nucleotides than of flavins. It is indeed just because of these differences that one may draw important mechanistic conclusions about flavoprotein catalysis from studies with deazaflavoproteins, as we want to show.

Abbreviations: FAD and dFAD, flavin- and 5-deazaflavin-adenine-dinucleotide; FMN and dFMN, flavin- and 5-deazaflavin-mononucleotide; RF and dRF, riboflavin and ribo-5-deazaflavin; DCIP, dichlorophenolindophenol

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Table 1 reveals several important differences between the native flavoproteins and the corresponding deazaflavoproteins. We shall attempt to provide explanations for these differences in chemical terms in the subsequent sections; for the moment we shall merely list them:

(1) In all cases the rate at which the deazaflavoprotein is reduced by substrate is at least 10^2 times lower, and in some cases as much as 10^6 times lower than the corresponding rate with the native enzyme.

(2) Apart from the single case of *N*-methylglutamate synthetase (and marginally the *Beneckea harveyi* flavin oxidoreductase) the deazaflavoproteins do not exhibit catalytic turnover.

(3) The capability of the reduced form to be reoxidized by oxygen, or artificial electron acceptors such as $Fe(CN)_6^{3-}$ or dichlorophenol-indophenol (DCIP) is lost with all of the deazaflavoproteins. Among the three main functions of flavoproteins
– (de)hydrogenation, O_2-activation and electron transfer (see below) –
the only one which is retained is that of the reversible dehydrogenation of the specific enzyme substrate.

Table 1
Comparative properties of native flavoproteins and their corresponding deazaflavin derivatives

Enzyme	Flavin	Rate of reduction by substrate	Rate of reoxidation by product	Rate of reoxidation by O_2	Reoxidation by other acceptors	Stability and colour radical	Ref.
Beneckea harveyi NAD(P)H: (flavin) Oxido-reductase	RF	1.0[a]	1.0[a]		Dihydro (deaza)flavins are liberated; reoxidation nonenzymic (cf. table 2)		[2]
	FMN	1.0[a]					
	FAD	0.25					
	dRF	0.0076	0.27				
	dFMN	0.0053					
	dFAD	no reaction					
N-Methyl-glutamate synthetase	FMN	20.8 min^{-1}	slow; rate limiting	fast		unstable[b]	[3]
	dFMN	0.17 min^{-1}	~ 13 min^{-1}			unstable	[4]
D-Amino acid oxidase	FAD	D-alanine ~ 10 000 min^{-1}	keto acid + NH_3 yields predominantly $E.FADH_2$: imino acid charge transfer complex	fast, > 1000 min^{-1}	$Fe(CN)_6^{3-}$ ~ 25 min^{-1}	red	[5] [6] [7]
		D-methionine ~ 100 000 min^{-1}					
		D-proline ~ 20 000 min^{-1}					
	dFAD	D-alanine 0.039 min^{-1}	90% reoxidation by 50 mM pyruvate + 50 mM NH_4^+	none		red	[1] [8]
		D-methionine 0.023 min^{-1}					
		D-proline 0.20 min^{-1}	k = 0.5 min^{-1}				[2]
Old Yellow enzyme	FMN	80 min^{-1}		~ 40 min^{-1}	$Fe(CN)_6^{3-}$ 160 min^{-1}	red	[9] [10]
(NADPH: oxygen oxidoreductase)	dFMN	0.03–0.8 min^{-1}	none	none	$Fe(CN)_6^{3-}$ none	unstable	[2]
Aspergillus niger glucose oxidase	FAD	~ 70 000 min^{-1}		1.26×10^8 M^{-1} min^{-1}	$Fe(CN)_6^{3-}$ DCIP, slow	blue or red pK 7.5	[11] [7]
	dFAD	~ 120 min^{-1}	none	none	DCIP, none	unstable	[2]

Table 1 (continued)

Enzyme	Flavin	Rate of reduction by substrate	Rate of reoxidation by product	Rate of reoxidation by O_2	Reoxidation by other acceptors	Stability and colour radical	Ref.
Mycobacterium smegmatis L-Lactate monooxygenase	FMN	14 000 min^{-1}	$E.FMNH_2$: pyruvate charge transfer complex	1.1×10^8 M^{-1} min^{-1} ($E.FMNH_2$: pyruvate) 5.4×10^5 M^{-1} min^{-1} (free enzyme)		red	[12] [13]
	dFMN	1.1 min^{-1}	rapid (complete) reoxidation $k > 100$ min^{-1}	none		unstable	[14]
Pig liver glycollate oxidase	FMN	$\geqslant$ 1250 min^{-1}		$\geqslant$ 620 min^{-1}	DCIP $\geqslant$ 1250 min^{-1}	red	[15]
	dFMN	0.08 min^{-1}	rapid; not quantitated	none	DCIP, none	unstable	[16]
Bovine milk xanthine	FAD	Xanthine 1100 min^{-1}	negligible	7200 min^{-1}	$Fe(CN)_6^{3-}$ 180 min^{-1}	blue	[17] [18]
oxidase	dFAD	Xanthine, none NADH, slow		none	$Fe(CN)_6^{3-}$ none	unstable	Massey unpublished
Flavodoxin[c]	FMN	reduced by NADPH plus ferredoxin-NADP reductase		$E.Fl_{red}H_2 \rightarrow E.FlH^{\cdot}$, fast $E.FlH^{\cdot} \rightarrow EFl_{ox}$, slow	$Fe(CN)_6^{3-}$ fast	blue	[19] [20]
	dFMN	not reduced by NADPH plus ferredoxin-NADPH reductase				unstable	[21] Massey unpublished

[a] The rates given are relative to that found for the reduction of riboflavin (RF) with NADH as reductant. As the flavin in this case is a substrate/product free in solution several column headings are not applicable

[b] Not reported, probably because of no radical stabilized, as in general for transhydrogenases

[c] The results quoted are for *P. elsdenii* flavodoxin (native protein) and *P. elsdenii* and *Azotobacter flavodoxin* (deazaflavoprotein)

(4) With the one exception of the red radical in D-amino acid oxidase, there is no stabilization of the deazaflavin radical state by the apoprotein. This should be contrasted with the almost universal stabilization of either the blue neutral or the red anionic radical in unmodified flavoproteins.

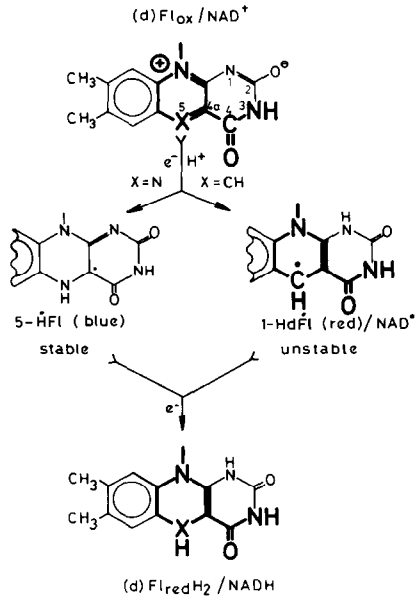
2. Comparison of the chemical properties of flavins, deazaflavins and nicotinamides

In table 2 are listed properties and chemical reactivities of flavins, deazaflavins and nicotinamides in their oxidized, $1e^-$- and $2e^-$-reduced states. In all three oxidation states it is clear that deazaflavins

Table 2
Comparative chemical properties of flavins, deazaflavins and nicotinamides

Property		Flavins (FMN)	Deazaflavins[a]	Nicotinamides[b]
Redox potential pH 7				
$1e^-$	ox/rad	− 0.240 V [22]	− 0.770 V [23]	− 0.850 V [25]
	rad/red	− 0.172 V [22]	+ 0.130 V [23]	+ 0.210 V [25]
$2e^-$:	ox/red	− 0.205 V [22]	− 0.320 V [23] − 0.275 V [24]	− 0.320 V [25]
Stability of covalent half-reduced dimer		none [26]	+++ [27]	+++ [28]
Attack by nucleophiles				
on oxidized form		+	+++	+++
K_d (sulfite)		0.95 M [29]	1×10^{-2} M [30] 3×10^{-3} M [31]	2×10^{-2} M [32]
K_d (cyanide)		no complex detectable [30]	3×10^{-3} M [30]	4×10^{-3} M [33]
Reduction by				
$Na_2S_2O_4$		fast ($1e^-$) [34]	slow [21]	slow [35]
$NaBH_4$		slow [36]	fast [37]	fast [38]
Reaction of $2e^-$-reduced forms with oxidants				
$1e^-$-oblig.	O_2[c]	very fast ($t_{1/2}$ ms-s) [39]	very slow ($t_{1/2}$ ~ days) [38]	none
	$Fe(CN)_6^{3-}$	$> 10^8$ $M^{-1}s^{-1}$ [39]	1.1 $M^{-1}s^{-1}$ [31]	slow [31]
	cytochrome *c*	$> 10^7$ $M^{-1}s^{-1}$ [31]	very slow ($t_{1/2}$ ~ h) [31]	very slow ($t_{1/2}$ ~ days) [31]
ambiguous $1e^-/2e^-$	DCIP	very fast [31]	5.7×10^2 $M^{-1}s^{-1}$ [31]	slow [31] (3.7 $M^{-1}s^{-1}$ *N*-dichloro benzyl Nic$^+$) [40]
	phenazine methosulfate	very fast [31]	4.2×10^4 $M^{-1}s^{-1}$ [31]	~ 5×10^2 $M^{-1}s^{-1}$ [31]
	Fl_{ox}	~ 5×10^8 $M^{-1}s^{-1}$ [41] ($1e^-$)	1.7×10^2 $M^{-1}s^{-1}$ [31] ($2e^-$)	0.16 $M^{-1}s^{-1}$ [42] ($2e^-$)
$2e^-$-oblig.	dFl_{ox}	not feasible thermodyn.	22 $M^{-1}s^{-1}$ [38]	(*N*-propyl Nic$^+$ 84 $M^{-1}s^{-1}$) [43]
				(*N*-propyl Nic$^+$ 12 $M^{-1}s^{-1}$) [43]
	NAD^+	not feasible thermodyn.	not reported	$\geq 2 \times 10^{-3}$ $M^{-1}s^{-1}$ [44]

[a] Deazariboflavin or deaza-FMN, unless specified
[b] Unless specified, results are for NAD^+
[c] Strictly speaking, O_2 is not a '$1e^-$-oblig.' acceptor toward normal flavin, since apart from the common $1e^-$ pathway, $H(d)Fl^{\ominus}_{red} + O_2 \rightarrow H(d)\dot{F}l + \dot{O}_2^-$, the flavin-specific reaction $H_2Fl_{red} + O_2 \rightarrow HFlOOH \rightarrow Fl_{ox} + H_2O_2$ must be considered [51] which is out of the present context



Scheme 1. Structural comparison of neutral (deaza)flavin and nicotinamide species in their three redox states: X = N, flavin; X = CH, deazaflavin; nicotinamide (Heavy lines).

resemble nicotinamides rather than flavins. This similarity, although expected for the fully reduced state on the basis of structure, also extends to the oxidized state, even though oxidized nicotinamides possess a full positive charge in contrast to the partial one in the center of deazaflavins (scheme 1).

3. Reactivity of the oxidized states

Reactions with the reducing agents, dithionite and borohydride, are strikingly different:
With flavins the dithionite reaction is rapid, proceeding mainly through 1e$^-$-reduction involving the radical $\dot{S}O_2^-$ [34]. With deazaflavins and nicotinamides, dithionite reduction is slow. In the case of nicotinamides a covalent C(4)–$SO_2^{\ominus}$ intermediate is rapidly produced prior to formation of the dihydronicotinamide which is split slowly to yield sulfite and reduced nicotinamide [35]. Thus the transfer must be conceived of as a 2e$^-$ process.

A similar sulfinate intermediate though less intensely colored, must be assumed for the reduction of deazaflavins, as shown by the biphasic kinetics [21].

With borohydride, 2e$^-$ reduction of deazaflavins and nicotinamides is rapid. In marked contrast is the very sluggish reaction of BH_4^- with flavins, where photoactivation is necessary for efficient reaction, and where reduction of the 4-carbonyl occurs in competition with the usual 1,5-reduction [36].

Even more instructive is a comparison of the susceptibility of the oxidized forms to nucleophilic attack. Addition compounds of nicotinamides with NH_2OH, CN^- and SO_3^{2-} are formed rapidly in a reversible equilibrium reaction [45]. Similar reactivity

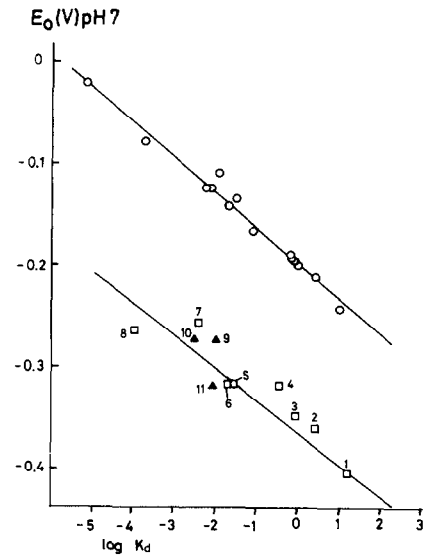


Fig.1. Linear free energy relationship between E'_0 pH 7 and dissociation constants for sulfite addition to flavins (upper line) (○), and nicotinamides, (□), and deazaflavins, (▲), (lower line). The data for flavins are from [29]. The K_d values given in that paper, $K_{(SO_3^{2-} + HSO_3^-)}$, are corrected to $K_{SO_3^{2-}}$. The remaining compounds are:
(1) *N*-Methyl nicotinamide, K_d from [32], E'_0 from [23]
(2) *N*-Benzyl nicotinamide, K_d from [32], E'_0 from [33]
(3) *N*-2,6 Dichlorobenzyl nicotinamide, K_d [32], E'_0 [33]
(4) NAD [47]
(5) NAD [48]
(6) NAD$^+$ [32]
(7) APNAD$^+$ [47]
(8) *N*-Tetraacetylglucose nicotinamide, K_d [32], E'_0 [33]
(9) Deazariboflavin, K_d from [16], E'_0 from [24]
(10) Deazariboflavin [31]
(11) Deazaflavin-3-sulfonate, G. Blankenhorn, unpublished data, cf. [23]

is found with deazaflavins (cf. table 2). In contrast, flavins, although susceptible to nucleophilic attack, require much higher concentrations of reactants to form detectable amounts of complexes; it is only in the case of sulfite that such complexes have been described with free flavins. Müller and Massey [29] demonstrated that the K_d of sulfite binding was directly related to the redox potential of the flavin. This relationship is shown in fig.1 for a wide variety of flavins. A similar linear free energy relationship can be constructed from literature values of sulfite–nicotinamide dissociation constants and the corresponding nicotinamide potentials. This is also shown in fig.1.

It is evident that if one compares at the same redox potential, the binding affinity of sulfite for nicotinamides is some 10^5-times greater than for flavins. Also shown in fig.1 are the K_d values for deazaflavin–sulfite addition compounds. These lie directly on the plot for the nicotinamides, providing strong evidence for the similarity of these two classes of compounds.

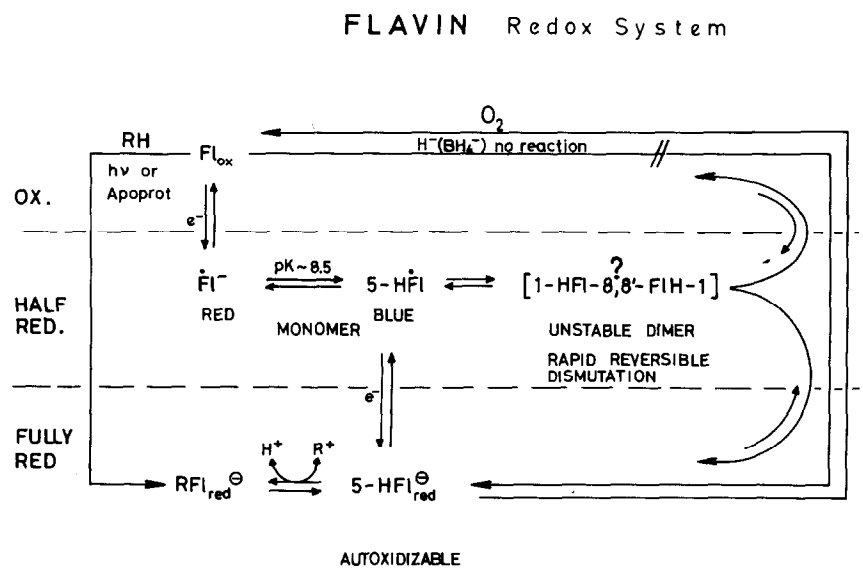
It is important to note that as indicated in scheme 2, direct $2e^-$ reduction of flavin by hydride transfer is only feasible photochemically or in the protein bound state. The very slow dark reduction of free flavin by NADH [46] is still much faster than flavin reduction by $NaBH_4$ [27].

4. Reactivity of the radical states

At least two forms of flavosemiquinones [49,50] are known to exist under physiological conditions:

Upon addition of a single electron to Fl_{ox}, the red radical anion $\dot{F}l^-$ is formed which, in the protein-free system, is protonated with a $pK \sim 8.5$ [50] to yield the blue neutral species 5-HFl, whose structure was established by comparison with the corresponding alkyl derivatives 5-RFl [49]. This blue radical is of moderate thermodynamic stability in the protein-free system. Its equilibrium of disproportionation is established rapidly [42], as pictured in scheme 2, based on data from the review by Hemmerich [51].

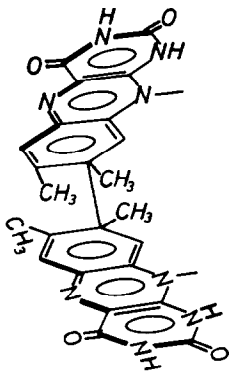
Favaudon [26] has shown that this dismutation reaction proceeds via a reactive dimer absorbing at 730 nm, not identical with the well known π-charge transfer complex $Fl_{ox} \sim Fl_{red}H_2$, which is detectable at high (> mM) concentrations and in aqueous solution only [52]. Various reasons outlined by Hemmerich [53] favor a covalent structure 1-HFl-8,8′-Fl^- (scheme



Scheme 2. Essential molecular species in the flavin redox system (neutral pH range) and pathways of Interconversion. For structures cf. schemes 1 and 3.

3.1) for this transient dimer. The fact that the 'inter-flavin' linkage in this species is between two carbon centers, should not be taken as an argument against its kinetic lability, as the well-known example of the less complicated alloxan–alloxantin–dialurate [54] redox system demonstrates (scheme 4) and where the CC-bond is cleaved rapidly and reversibly in the disproportionation of the half reduced dimer.

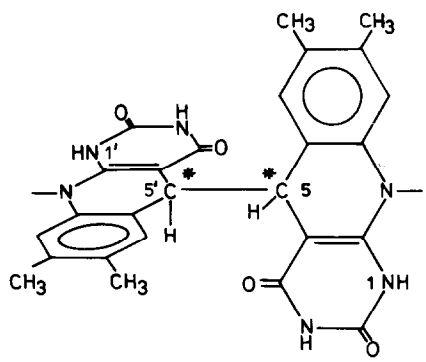
The blue radical species 5-HF̈l can be further stabilized chemically by alkylation, as shown with the derivatives 5-RF̈l [49] or biochemically via binding to a suitable apoprotein, as found in the wide-spread



$(FlH)_2$ ≡ 1-HFl-8,8'-FlH-1

$Fl_{ox} + Fl_{red}H_2 \rightleftharpoons (FlH)_2 \rightleftharpoons 2\,\dot{F}lH$

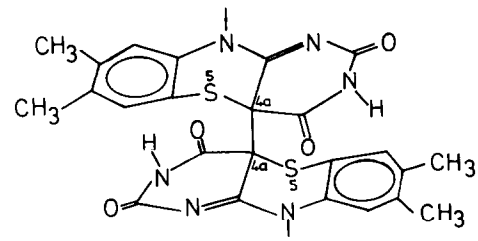
Scheme 3.1.



$(dFlH)_2$ ≡ 1-HdFl-5,5'-dFlH-1

* asymmetric centers

Scheme 3.2. $(dFlH)_2 \overset{h\nu}{\rightleftharpoons} 2d\dot{F}lH$



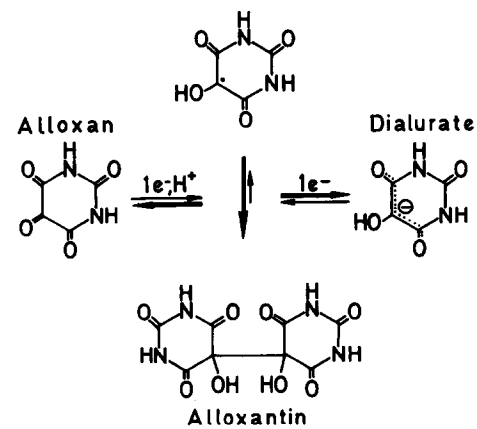
$(sFl)_2$ = sFl-4a,4a'-sFl

Scheme 3.3. $(\bar{s}Fl)_2 \rightleftharpoons 2\,s\dot{F}l$

Scheme 3. Structure and stability of radical dimers: 3.1, flavins; 3.2, deazaflavins; 3.3, thiaflavins.

group of 'blue radical flavoproteins' [7,9]. In all cases where flavin radicals could be shown to act as catalytically essential intermediates, the radicals are of the blue variety 5-HF̈l, and the stabilization is thought to be brought about by a regiospecific hydrogen bridge pointing from the apoprotein towards N(5) (see below).

On the other hand, if the lone pair in the flavosemiquinone anion $\dot{F}l^-$ is not blocked at N(5), but at N(1) or O(2α), be it chemically by way of alkylation to



Scheme 4. Alloxan–dialurate redox system based on the most recent data [54]. Note that the CC-dimer alloxantin largely disproportionates in solution in a rapid and reversible reaction.

yield 1- or 2α-RḞl, no drastic spectral difference is observed between anionic and neutral radical. By stabilization through hydrogen bonding to these same centers, it is possible that a similar situation may exist in flavoprotein red radicals. Hence, as mainly disregarded in the earlier literature, red flavoprotein radicals can either be anions Ḟl$^-$ or neutral tautomers of the type 1- or 2α-RḞl, and a clear distinction is only possible in those few cases where a radical pK is seen in the protein-bound state, such as glucose oxidase [7] and lysine monooxygenase [55]. Furthermore, whenever a flavoprotein exhibits a red radical, it appears that the radical state is biocatalytically non-essential, i.e., the red radical cannot be generated by the action of substrates, but only artificially.

In the free chemical system, e.g., 2α-RḞl, thermodynamics are strictly in favor of dismutation [56]. Thus the radical state is destabilized upon blocking the N(1)- or O(2α)-lone pair, while it is stabilized upon blocking the N(5)-lone pair.

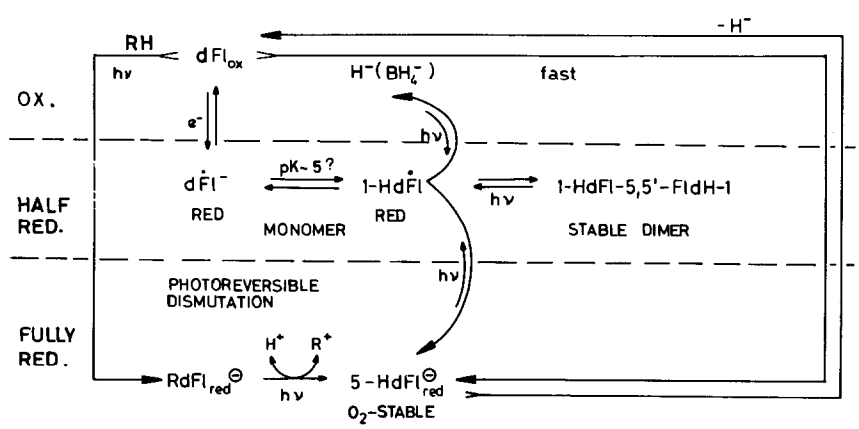
When considering 5-deazaflavins, it is obvious that the radical anion dḞl$^-$ will, unlike normal flavin, not accept a proton in position 5, now modified from N to CH, but instead in position 1. Furthermore, a species 5-HdḞl, having a sp^3-carbon at position 5, would, unlike 5-HḞl, not permit efficient tricyclic conjugation and spin delocalisation within the chromophore, which clearly renders 5-HdḞl extremely unstable as compared to 5-HḞl (schemes 1 and 5).

Hence, with 5-deazaflavins, we have to expect a 'red radical system' with the following properties:

(1) No drastic change of absorption between the neutral and anionic radical species. This has been verified for flavins [57].

(2) Drastic thermodynamic destabilization of the radical state and thus no catalytically essential radicals. These predictions are borne out by the following experiments:
In order to generate 5-deazaflavin radicals at all, we have to employ enforced one-electron reduction methods. Thus pulse radiolysis by hydrated electrons as well as $\dot{C}O_2^-$-radicals generated through the reaction of formate + ȮH, yields a transient red radical absorbing around 530 nm over the whole range of pH 4–10, which decays by disproportionation (cf. scheme 5) with a yield of >90% [58].

Photochemically, 1e$^-$-reduction can only be enforced by the 'self-reaction' within the deazaflavin



Scheme 5. Essential molecular species in the deazaflavin redox system (as compared to flavin, scheme 2) and pathways of interconversion. Note that among the two possible ways of photochemical radical formation, the comproportionation works at 400 nm, while the photodissociation of dimer requires 320 nm, unless it is photosensitized by dFl$_{ox}$. It cannot be excluded that the comproportionation would lead directly to the dimer, whose dissociation might be the only way to generate the radical. The reaction pathway '-H$^-$' is meant to be realized by all 2e$^-$-oxidants.

system, while extraneous photo-substrates lead to direct formation of reduced adducts $RdFl_{red}H$ (scheme 4) [27]. These adducts may then undergo secondary 'self-reaction' more or less readily, depending on the nature of R, according to the equation

$$RdFl_{red}H + dFl_{ox} \xrightarrow[h\nu]{H^+} 2\ d\dot{F}lH + R^+$$

The final product of photochemistry is the stable and crystallisable dimer 1-HdFl-5,5′-dFlH-1 [27] (scheme 3.2).

In agreement with this structure, two diastereomers can be distinguished, one of which accumulates upon prolonged irradiation. This is evidence for dFl_{ox}-sensitized photodissociation and (dark) reformation of the dimer, as given by the equation:

$$dFl'_{ox} + (5,5'\text{-HdFl})_2 \underset{h\nu\ 400\ nm}{\longleftrightarrow}$$

HdFl-5,5′-dFl′ H + dFl_{ox} (cf. scheme 3.2)

from which once again it follows that the deazaflavin radical must have the tautomer structure 1-Hd$\dot{F}$l (and not 5-Hd$\dot{F}$l). We have further corroborated the occurrence of photodissociation by use of a labelled sensitizer dFl'_{ox} and we find incorporation of the labelled deazaflavin into the dimer [27].

Thus it is shown that deazaflavin radical dismutation is irreversible in the dark – in contrast to flavosemiquinone dismutation – but easily reversible in the light.

Scheme 4 contains one point of inconclusiveness: The final product of pulse radiolysis is $dFl_{red}H_2$ (via radical dismutation), while photochemistry yields almost quantitatively the $1e^-$-reduced system in the form of the dimer $(HdFl)_2$, though both processes run through the same radical intermediates 1-Hd$\dot{F}$l or d$\dot{F}l^-$. We have, therefore, checked a third independent method of $1e^-$-reduction, i.e., controlled voltage electrolysis at $\leqslant -770$ mV, corresponding to the dFl_{ox}/d$\dot{F}$l-potential (table 2). Independent of the environment – i.e., in CH_3CN with $NEt_4^+Br^-$ as electrolyte as well as in water with NH_4^+-acetate, pH 4–9, we obtain 100% formation of the dimer $(dFlH)_2$ with no further reduction to the dihydro state [27].

Hence, we can compare the two methods of dark reduction, radiolysis and electrolysis, the first one yielding dismutation, the second one dimerization of the same radical, irrespective of whether its state of protonation is d$\dot{F}l^-$ or d$\dot{F}$lH. The only way out of this dilemma is by the conclusion that there are at least two types of covalent dimers, namely the well-established colorless, stable and crystallisable 5,5′-dimer which is analogous to $(NAD)_2$ and a flavin-analogous [26], colored, labile 8,8′-dimer leading to dismutation (scheme 3.1). The following hints for the occurrence of this competitive 8,8′-dimerization can be presented:

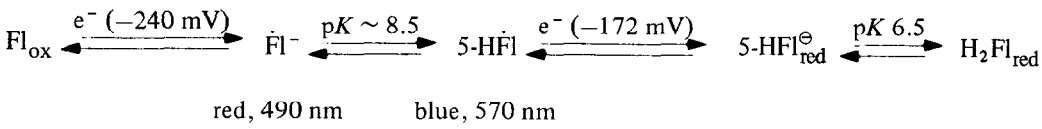
(i) The otherwise analogous nicotinamide radical NAD$^\cdot$ yields the same decay, i.e., 100% irreversible dimer formation by both methods, radiolysis and electrolysis [28] since it lacks a position 8 and, therefore, the opportunity to form a covalent radical dimer which can redissociate easily. In this respect nicotinamide and deazaflavin behave in different ways, but this difference is the one of the few and only minor in nature (see below).

(ii) In deazaflavin photoreductions by e.g., EDTA as donor, a relatively long-lived by-product absorbing in the range 360 nm is invariably observed and can be characterized by its fluorescence emission at 430 nm, and excitation maxima at 303, 365 nm [31] by which it differs from the 5,5′-dimer (emission max 375 nm, excitation max 310 nm) and the starting dFl_{ox} with its strong emission max at 460 nm, excitation max 318, 390 nm. These values have been found for 7,8-unsubstituted deazaflavin. When positions 7 and 8 are methylated, these intermediate by-products are much less stable (in agreement with an 8,8′-dimer structure) and their fluorescence properties are difficult to evaluate.

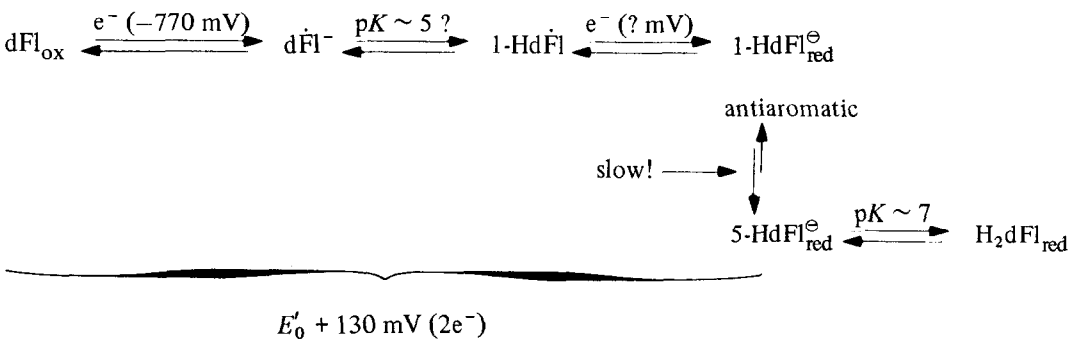
As outlined by Hemmerich [53], the decay of the hypothetical 8,8′-dimer with formation of the fully reduced species 1,5-(d) $Fl_{red}H_2$ (cf. scheme 1) depends on the availability of protons to add in position 5. This would explain why in free solution, i.e., by radiolysis, dismutation is favored, while at an electrode surface 5,5′-dimerization prevails, which can be obtained by direct reaction of the anions:

$$2\ d\dot{F}l^- \longrightarrow (5,5\text{-dFl})_2^{2-} \text{ (cf. scheme 3.2)}$$

Flavin



Deazaflavin

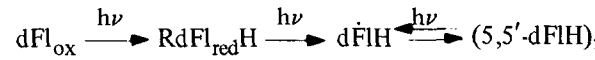


Scheme 6. Sequence of steps in the 1e^--reduction (electron plus proton uptake) for the flavin and the deazaflavin system.

The sequence of events occurring upon reaction of flavin and deazaflavin, respectively, with single electrons followed by protons, is outlined accordingly in scheme 6. From this it can be concluded:

(i) For flavins the sequence of events is transfer of an electron, followed by protonation at position 5 to yield a stable radical. Transfer of the second electron and the subsequent protonation occur at the position of highest basicity and are rapidly reversible.

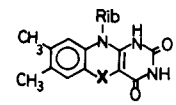
(ii) For deazaflavins the sequence is electron transfer, protonation at position 1 yielding an energy-rich radical, followed by dimerization which is irreversible if occurring in position 5. In order to obtain further reduction, the proton must add to C(5), which is kinetically and thermodynamically unfavorable for the radical monomer and thus might only occur after 8,8′-dimerization with concomitant dismutation of the dimer, which is irreversible in the dark. Because of the facile formation of deazaflavin radicals in the photochemical reactions – in the sequence



shown in scheme 5 – and owing to the low redox potential (table 2) and high reactivity of the radicals, deazaflavins could be shown to act as very efficient catalysts in the photoreduction of a wide variety of redox carriers, including flavoproteins, heme proteins and iron-sulfur proteins [59].

The difference between flavins and deazaflavins is, therefore, mainly due to destabilization of the blue radical 5-H$\dot{F}$l because of substitution of N(5)H by CH_2. It is clear, that a high spin density can be induced in an NH-group by electron withdrawal from the non-bonding sp^2- or π-bonding p_z-orbital, while in a sp^3-methylene group this is not feasible. It could, therefore, easily be imagined that replacement of N(5)H by sulfur would lead to the contrasting case of radical stabilization and, concomitantly, enforcement of 1e^--transfer reactions. We have shown [60] that this is the case with the so-called '5-thiaflavins' (scheme 1, X = S^+). Here we observe a stable radical

Table 3
Correlation of main activities and radical properties for nicotinamides, deazaflavins, flavins and thiaflavins



X = NH, $Fl_{red}H_2$ (Flavin)
X = CH_2, $dFl_{red}H_2$ (Deazaflavin)
X = S, $sFl_{red}H$ (Thiaflavin)

	NAD	Deazaflavins	Flavins	Thiaflavins
Radical state structure	NAD·	1-HdFl·	5-HFl·	SFl·
Stability				
Chemical system { Dimer.	Irreversible	Photoreversible	Dimer short-lived	Reversible
Chemical system { Disprop.	Not observed	Photoreversible	Reversible	Irreversible
Protein bound	Unstable	Unstable	Stable	Very stable
Activities				
(De)hydrogenation ($2e^-$)	++	+	++	–
O_2-Activation	–	–	++ ($2e^-$ or $1e^-$ fast)	+ (slow e^-)
e^--Transfer	–	–	++	+
Active redox shuttles				
$2e^-$	+	+	+	–
Upper $1e^-$	–	–	+	–
Lower $1e^-$	–	–	+	+

Note that from left to right pure hydride transfer is changing into pure electron transfer

sFl· in equilibrium with a labile, but crystallisable 4,4′-dimer (scheme 3.3). Unfortunately, the $1e^-$-potential $sFl_{red}H/sFl$· is found to be as high as +400 mV. Consequently, apoflavodoxins as pure electron transferases will bind thiaflavin and stabilize the radical sFl· further, and the $1e^-$-shuttle will work well in the thiaflavodoxin, but not at the low potential level required for biological activity [60]. We are trying to overcome this difficulty by introduction of electron-donating +M-substituents.

In table 3, we have summarized the correlation between (co)enzyme activity and radical stability for the whole range from the '$2e^-$-only' nicotinamides to the '$1e^-$-only' thiaflavins, via deazaflavin, which is $2e^-$-only in the ground state and either $1e^-$ or $2e^-$ in photochemistry. Native flavin is much more versatile, employing $1e^-$- or $2e^-$-transfers in either the ground or excited state, the actual choice being taken by the apoprotein.

5. Reactivity in the reduced state

It is also obvious from table 2 that the similarity in chemical reactivity between nicotinamides and deazaflavins extends to the reduced state. Both are reoxidized very slowly by O_2, whereas reduced flavins are oxidized rapidly. Reduced flavins also react at close to diffusion-controlled rates with the obligatory $1e^-$-acceptors, $Fe(CN)_6^{3-}$ and cytochrome *c*, whereas dihydrodeazaflavins and dihydronicotinamides react much more slowly by a factor of $\geqslant 10^7$ with these oxidants. With the ambiguous $1e^-/2e^-$-acceptors, dichlorophenol indophenol and phenazine methosulfate, reoxidation rates with dihydrodeazaflavins are intermediate between those with reduced flavins and nicotinamides.

In the last three columns of table 2 are listed the rates of redox transfer between the three groups of compounds, including the self-reaction rates. Again

deazaflavins are intermediate between flavins and nicotinamides in these rates. The rate of flavin disproportionation ($Fl_{ox} + Fl_{red}H_2 \rightleftharpoons 2\,\dot{F}lH$) is very rapid, close to diffusion controlled [42]. The analogous reaction with deazaflavins, which does not appear to involve radicals, but pure hydride exchange has been measured with ^{14}C-labeled deazaflavins and found to be quite slow (22 $M^{-1}s^{-1}$) (cf. table 2). The interchange between oxidized and reduced nicotinamides has been measured with ^{3}H-labeled compounds. Hence, the value listed in table 2 ($2 \times 10^{-3}\,M^{-1}s^{-1}$) is a minimal one, since a kinetic selection against ^{3}H-transfer is to be expected. The oxidation rates of dihydrodeazaflavins and NADH by oxidized flavin differ by a factor of 10^3; however this difference largely disappears if a simple nicotinamide model, *N*-propyl dihydronicotinamide, is compared. Quite similar rates of exchange of reducing equivalents are found between $dFl_{red}H^-$ and dFl_{ox} and between *N*-propyl dihydronicotinamide and dFl_{ox}.

Thus it appears that deazaflavins and nicotinamides behave as $2e^-$-agents towards themselves and are unambiguous donor–acceptors, also towards flavin, while the flavin is ambiguous; the self-reaction is $1e^-$-transfer in the free state, while in the bound state the distinction between $1e^-$- and $2e^-$-transfer modes is dictated by the substrate and the apoprotein (see below).

6. The mechanism of flavoprotein action as studied by deazaflavoproteins

It has long been established that the apoproteins of flavoenzymes can recombine not only with their natural flavins, but also with many coenzyme derivatives modified in the isoalloxazine moiety (see for example studies with D-amino acid oxidase [61], Old Yellow enzyme [62], flavodoxins [63] lipoyl dehydrogenase [64] NADPH–cytochrome *c* reductase [65]. It is evident from these studies that the chief factor determining the binding of flavin to apoprotein is the nature of the N(10)-side chain. Thus for FAD-enzymes only modified flavins at the FAD level will bind to the apoprotein while with FMN-enzymes modified flavin will bind only at the FMN level. This applies equally well for deazaflavins. Since deazaflavins exhibit a shape closely similar to the normal flavin and since they are bound firmly to the enzyme active site mainly by virtue of their side chains it is not surprising, that at least some catalytic activity should be retained in the deazaflavo-holoprotein. The real question which has to be faced is whether the modified enzyme retains the full spectrum of natural activities and to what extent.

Flavins are particularly versatile coenzymes, whose spectrum of activities is even enlarged upon binding to proteins. According to the state of knowledge in 1976 [51] three main types of flavoprotein activities can be distinguished by their chemical as well as enzymological characteristics, namely reversible substrate dehydrogenation, electron transfer and activation of molecular oxygen. Only the two latter activities are retained efficiently in the free flavocoenzymes upon detachment from the protein. The reverse is true for free deazaflavins, which retain only a (faint) capability of dehydrogenation, reminiscent of free nicotinamide.

Furthermore, flavoproteins can be subdivided according to whether they act only in one of these three principal modes, or whether they combine two of them. Hence, we want to differentiate four main classes of flavoproteins:

1. *Transhydrogenases*
   Pure (de)hydrogenases or in better terminology, transhydrogenases. A typical example is the pyridine nucleotide transhydrogenase of *Azotobacter vinelandii* [66] which is active with NAD and NADP but reacts only slowly with oxygen and for which no natural acceptor for single electrons from the reduced enzyme is known. This enzyme fails to yield a stabilized semiquinoid form, even on photoreduction with EDTA.

2. *Pure e^--transferases*
   Pure e^--transferases, for which the widespread group of flavodoxins is representative. They are well established to shuttle between half-reduced and fully-reduced state under natural conditions.

3. *(De)hydrogenase-oxidase and -oxygenases*
   (De)hydrogenase-oxidase and -oxygenases efficiently combining activation of a CH-bond as input and activation of O_2 as output activity. Both activities circumvent the radical state [51].

4. *(De)hydrogenases-electron transferases*
(De)hydrogenases-electron transferases, transforming $2e^-$-redox equivalents from CH-substrate into two radical electrons or vice versa. The main flavoproteins of the chloroplast photosynthetic chain as well as the mitochondrial respiratory chain and the microsomal oxygenative apparatus are of this latter type. This type reacts with oxygen in general only slowly and, typically, with production of superoxide rather than peroxide.

This classification could in the last years be correlated with the type and stability of radicals observed in these enzyme groups at half reduction:
All flavoproteins which are meant by nature to deal with single electrons, i.e., types 2 and 4, invariably show blue radicals of at least moderate (type 4) or very high (type 2) stability, which are catalytically essential [1].

Group 1 enzymes do not appear to stabilize any radical, in keeping with the essential $2e^-$-nature of the reactions they catalyse while group 3 enzymes stabilize red radicals, which however appear to be catalytically inessential, since the radicals have not been observed to arise during catalysis, but only on reaction with artificial reductants. Thus it can be concluded that transhydrogenase as well as oxidase/oxygenase activities probably do not involve single electron steps.

From an inspection of the properties of deazaflavoproteins as laid out in table 1 it is clear that the deazaflavin modification leads to loss of two main activities, namely oxygen activation and electron transfer. Among the natural activities that of transhydrogenation alone is retained, but at a level which is in most cases far inferior to that shown by the natural enzyme. Scola-Nagelschneider et al. [67] have pointed out recently that problems of the mechanism of flavin action can be summed up in four questions:

1. Does a given flavoprotein activity involve transfer of $1e^-$- or $2e^-$-equivalents?

2. If $2e^-$-equivalents are transferred, is the now mandatory carrier a hydrogen in the form of a hydride unit or a carbon residue in the form of a carbanion?

3. If there is carbanion transfer, does the link between substrate and flavin have σ-covalent or π-charge transfer character?

4. If there is a σ-covalent intermediate, which is the site of fixation at the flavin nucleus (C)4a, N(5) or C(8)?

Probable solutions to these questions for flavins, deazaflavins and nicotinamides are collected in table 4.

Table 4
Problems pertinent to the mechanism of (deaza)flavin and nicotinamide action

Question		Answers		
		NAD	dFl	Fl
1	$1e^-/2e^-$ transfer ?	$2e^-$ only		either ($2e^-/2 \times 1e^-$ transform)
2	H^-/R^- transfer ?	either (H^-/R^--transformation)		R^- only
3	σ/π-bonded adducts ?	σ-bonded		either (σ/π-transform)
4	Site of covalent substrate fixation ?	C(4) ≡ C(5)		C(4a) or N(5) ↓↑ ↓↑ R^- R^+ (input) (output)

For the present evaluation of deazaflavins it is obvious that the first question must be answered in favor of $2e^-$-transfer, the deaza-modification thus turning the ambiguous flavin agent into an unambiguous $2e^-$-agent. Even if the first question were to be answered in favor of single electron transfer for a given flavoprotein activity, since flavin is an ambiguous agent meant by nature in many instances to mediate between $1e^-$ and $2e^-$-transfer, questions 3 and 4 still apply and should be considered thoroughly.

The crucial question, however, in the present context, is question 2. In the case of natural flavin-dependent dehydrogenation, many facts speak in favor of carbanion transfer [68–71]; for nicotinamides on the other hand, it is well established that their reaction with alcoholic substrates is a hydride transfer. This hydride input does not allow one, however, to conclude that hydride transfer is the output reaction of nicotinamide towards flavin. On the contrary, it seems that nicotinamides are unique as hydride acceptors, while at the same time they transform the hydride unit into something which is more easily dealt with by the flavin acceptor. Now deazaflavin being a flavin-shaped nicotinamide, the question is how will it behave?

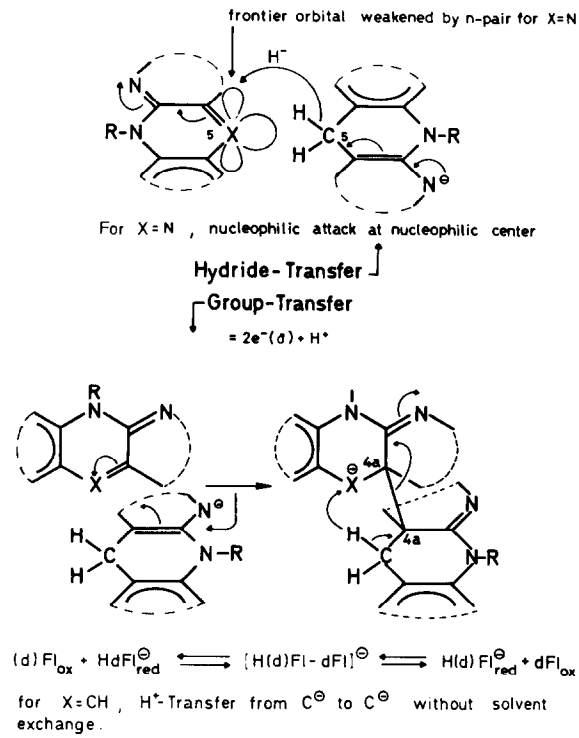
The solution of this problem is coincident with the question as to the mechanism of redox transfer between nicotinamide and flavin. Unlike other flavin substrates nicotinamide cannot be activated to yield a carbanionic unit, free or bound, since such an activation would run through an intermediate delocalized 8π-system of antiaromatic character. On the other hand, hydride abstraction by flavin from nicotinamide appears unlikely owing to the fact that the acceptor position at the flavin nucleus is a nitrogen of pyridine type, though of very low basicity, and such nitrogen centers can hardly be anticipated to catalyse the acceptance of a nucleophile. This is in agreement with the failure of flavin to accept hydride units from borohydride as a well established hydride donor which reacts rapidly with nicotinamide (cf. table 2).

Blankenhorn [23] has shown in his elegant work on this subject that up to now one cannot differentiate experimentally between hydride transfer, on the one hand, and a transfer of an electron pair through a covalent σ-bond, accompanied by proton transfer, on the other hand. Even the fact that no proton exchange with the environment takes place does not help to discriminate between the two possibilities, since an intramolecular proton shift within a covalent adduct of two redox partners would allow the H-label to be retained provided the H^+-transfer occurs between two carbon centers.

This situation is exemplified in scheme 7 showing the two alternatives of $2e^-$-transfer, namely hydride transfer (upper part) and $2e^-$ (σ) and proton transfer (lower part). We want to postulate, that the upper scheme describes the redox transfer between partners with X = CH, while the lower scheme applies, when at least one species with X = N is involved. Replacement of CH by N lowers at the same time the activation energy of the protein-free system.

Hence, question 2 may be answered in the present context by the statement that replacement of flavin-N(5) by CH in flavoproteins changes the (de)hydrogenation mechanism from $2e^-$ (σ) + H^+ towards a much less efficient, true hydride transfer.

This suggestion would at the same time answer



Scheme 7. Group versus hydride transfer with (deaza)flavins (d)Fl stands for either flavin or deazaflavin.

the above question 3 in favor of a 'σ-transfer'. There remains the question, why protein-bound deazaflavin would not accept carbanions in contrast to free deazaflavin which reacts rapidly, but irreversibly at C(5). This fact lends further support to the hypothesis put forward by Hemmerich and Jorns [72], in answer to question 4:
The flavin input site for carbanions and nucleophiles in general is position 4a, while in nicotinamides it is C(4), corresponding to flavin position 5. If, therefore, flavin is replaced by deazaflavin in a protein, carbanion transfer is cut off, since C(4a) has lost its acceptor properties.

Reviewing the function of the 'carriers' within redox chains, it can be concluded that, from alcoholic substrates, nicotinamide accepts hydride equivalents transforming them into 'carbanion' (or, more precisely $2e^-$ (σ) + H^+) equivalents to be accepted by flavin, which in turn can split them up to yield radical electrons. Both 'transformer' qualities are unique for the given coenzyme; thus flavin cannot deal with hydride, nor can nicotinamide deal with single electrons. Certain substrates, such as alcohols, can, however, be dehydrogenated in either way, namely by hydride as well as by carbanion transfer. A neighbouring carbonyl group will help the apoprotein to deprotonate a CH-substrate, thus flavoprotein substrates are generally but not mandatorily 'activated' in the sense $C^{\delta-}-H^{\delta+}$.

There remains the question of how the coenzymes receive the information on the way in which they have to act. For nicotinamides it is clear that they have to bind to two strictly different classes of proteins, namely to NAD-dependent dehydrogenases as coenzymes and to flavoproteins as substrates and that they have an absolute requirement for flavin as acceptor, if ever their reducing equivalents should reach a single electron (cytochrome) chain.

For flavins the question is more difficult, since $1e^-$- and $2e^-$-processes may have to occur within the same protein. Here it is likely that the type of activity shown is steered by hydrogen bridges directed from the apoprotein towards either N(5) for $1e^-$-transfer or to the N(1)/O(2α)-region for $2e^-$-transfer. This latter case leaves the C(4a)=N(5)-subgroup free for substrate addition, while the single electron transfer seems to occur not only through position 4a or 5, but maybe preferably C(8) [53]. This concept is in agreement with the fact that only this position is exposed to the environment in flavodoxins.

If a given flavoprotein behaves as a transformase, i.e., if it catalyses both dehydrogenation (input) and electron transfer (output) in respiration or vice versa in photosynthesis, the 'proton steering' must change in the course of these events by conformational rearrangements of the apoprotein skeleton.

In the light of this review, deaza-FAD xanthine oxidase is a most informative case of a deazaflavoprotein and deserves special comment. It has been demonstrated with the native enzyme that internal electron transfer between the molybdenum, FAD and iron–sulfur centers is a rapid $1e^-$-process, with the relative concentration of the reduced redox species being determined simply by their redox potentials and the total number of reducing equivalents in the enzyme [17,18]. When deaza-FAD is incorporated into the enzyme in place of FAD, and the enzyme incubated anaerobically with xanthine, there is rapid reduction of molybdenum and the iron–sulfur centers, but no reduction of deaza-FAD, even over a period of 2 days. That this is not due to a lowering of the potential of dFAD to unreachable levels is shown by the fact that NADH slowly reduces all three chromophores. Thus it can be concluded, as expected from the properties of free deazaflavins, that $1e^-$-transfer to the bound dFAD is not possible, but that NADH, by its innate capacity for $2e^-$-transfer is capable to reduce the bound dFAD and that slow electron transfer to the other centers occurs, possibly via the Mo-center, which could mediate between the $2e^-$ dFAD and the $1e^-$ iron–sulfur centers. Thus nature appears to have two methods to transform $2e^-$-equivalents into single electron equivalents, i.e., flavin and molybdenum.

Summarizing, deazaflavin is not a 'flavin-analog', but a 'flavin-shaped nicotinamide', which blocks flavin-dependent e^--transfer and O_2-activation and turns flavin-dependent transhydrogenation in a hydride transfer reaction. The very importance of deazaflavin is however, to be seen in the instability of its radical as generated photochemically [59] which provides a potent, but still clean and mild 'flavin-shaped' reductant in the hands of the biochemist. Scherings et al. [73] were first to use this tool for the specific reduction of the azotobacter nitro-

genase system, thus replacing the unspecific and unclean dithionite reagent, which throws light upon the versatility of this method.

Acknowledgements

We are greatly indebted to several colleagues for the permission to quote recent unpublished data, in particular to Drs I. Pecht and M. Goldberg, The Weizmann Institute of Science, Rehovot (Israel) for communicating the data on deazaflavin radiolysis, and from the Konstanz (FRG) group to Dr G. Blankenhorn for electrochemical and to Dr W.-R. Knappe for photochemical results to be published in detail elsewhere.

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