in the migrating carbon.[48] In these cases the bond-making alternative may resemble the species involved in the solvolysis of allylcarbinyl derivatives and may be more stable than the bond-breaking alternative (Figure 13). Clearly work needs to be done to understand these reactions and to determine if the analysis above is appropriate.

Concluding Remarks

Through the use of α secondary deuterium isotope effects, the extent of bond breaking and bond making in the transition state for the concerted 3,3 shift of 1,5-hexadienes has been examined. Remarkably, the KIEs reveal a dramatic change in transition-state structure upon substitution in a manner consistent with expectations based on Hammond's postulate (Thornton's parallel effect) and on Thornton's perpendicular effect. Diagramatic representation of this behavior in terms of a More O'Ferrall–Jencks energy surface has led to a quantitative relationship between the activation free energy for various 3,3 shifts and the activation energies for the bond-breaking and bond-making nonconcerted alternatives that appears to be the concerted reaction analogue of the Hammett equation.

Studies of some degenerate 1,3 shifts indicate that though Woodward–Hoffmann allowed stereochemistry is observed, suggesting concert, the rearrangement is best interpreted in terms of least motion generation and closure of a biradical intermediate. Whether or not other 1,3 shifts are also nonconcerted will require execution of experiments designed by Dolbier to reveal a two-step process. Analysis of 1,3-, 1,5-, and 2,3-sigmatropic shifts in terms of the relative amounts of bond breaking and bond making provides an insight to mechanism that indicates the direction of further work.

I thank the donors of the Petroleum Research Fund, administered by the American Chemical Society, for initial support of the work described here and the National Science Foundation for generous continuing support. The skilled graduate students who performed the research also supplied many of the insights necessary to place their work in perspective given here. Thanks also go to Eric Otterbacher and Mike Warner for their help in developing nonlinear free-energy relationships, to Professor John Bartmess whose storehouse of gas-phase data and insights into quantitative chemical relationships have continually stoked our interests, and to Professors Jack Shiner and Dick Schowen whose analyses of solution reactions have inspired some of the experiments and interpretations provided in this Account.

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Flavin Coenzymes: At the Crossroads of Biological Redox Chemistry

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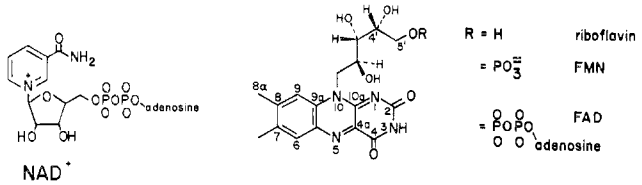
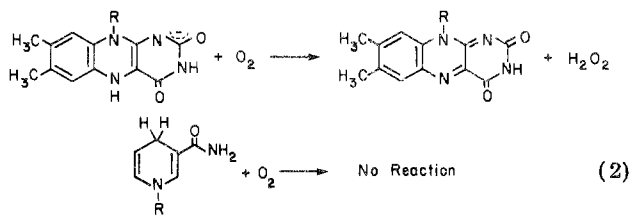
Nature and Scope of Flavoenzyme Redox Catalysis

Riboflavin, vitamin B_2, is a yellow tricyclic (isoalloxazine) molecule which in biological systems is phosphorylated and then adenylylated further to the two active redox coenzyme forms FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide). Flavins and nicotinamide coenzymes (eq 1) (NAD/



(1)

NADH, NADP/NADPH) are the primary acceptors for electron pairs removed from the vast majority of functional groups in soluble metabolites undergoing cellular oxidation or, in the reverse direction, reduction. Their substrates include amines, amino acids, alcohols, sugars, hydroxy acids, dithiols, aldehydes, ketones, and acids. These oxidations are net two-electron redox processes, and in a stoichiometric sense either flavin or nicotinamide coenzymes can be used for the same reaction. However, the tricyclic flavins are much more versatile catalysts than the monocyclic nicotinamides, as evinced by two related properties: (a) nicotinamides appear restricted to two electron pathways while flavins can undergo both facile two-electron and one-electron chemistry; (b) dihydronicotinamides are inert to oxygen, but dihydroflavins, with a central dihydropyrazine ring system, are highly reactive for reductive oxygen metabolism (eq 2). These chemical features place flavin



(2)

coenzymes squarely, and uniquely, at the crossroads of

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biological redox chemistry. On the one hand, they serve as step-down transforming redox switches between obligate two-electron donors (e.g., oxidation of NADH → NAD, succinate → fumarate, lactate → pyruvate) and obligate one-electron acceptors (iron–sulfur cluster proteins or heme proteins) in cellular redox metabolism. On the other hand, the reactivity with molecular oxygen, a probable consequence of flavin semiquinone stability and kinetic accessibility in biological systems, allows flavins to serve as cofactor for both net two-electron reduction of O_2 to H_2O_2 and four-electron reductive activation and cleavage in monooxygenation reactions (eq 3). Both are key processes in aerobic

$$XH_2 + O_2 + SH \xrightarrow{\text{E-flavin}} X + H_2O + SOH \quad (3)$$

XH_2 = reductant; SH = substrate

organisms.[1-3]

Reductive and Oxidative Half-Reactions

Another feature which distinguishes flavoenzymes from most simple nicotinamide-requiring dehydrogenases is a physical parameter. Flavoenzymes exist as nondissociable, but generally noncovalent, complexes of apoprotein and flavin coenzyme while nicotinamides are released freely at the end of each catalytic cycle and so are consumed as *redox cosubstrates* in any turnover (eq 4). In contrast, a flavin

$$NAD^+ + SH_2 \xrightleftharpoons{\text{Enz}} NADH + H^+ + S \quad (4)$$

cofactor that undergoes, e.g., a two-electron reduction in a catalytic cycle stays tightly bound at the enzyme's active site and must be returned to its original two-electron oxidized oxidation state before the holoenzyme molecule can carry out another catalytic cycle. There must be not only a reductive half-reaction but also an oxidative half-reaction to regenerate active catalyst (eq 5). Thus, flavins do not appear *explicitly* in the redox

$$SH_2 + \text{Enz–FAD} \rightleftarrows S + \text{Enz–FADH}_2 \quad \text{reductive half-reaction}$$

$$\text{Enz–FADH}_2 + X \rightleftarrows XH_2 + \text{Enz–FAD} \quad \text{oxidative half-reaction}$$

$$SH_2 + X \rightleftarrows S + XH_2 \quad (5)$$

stoichiometry, analogous, for example, to the role of bound pyridoxal phosphate in transaminations or of thiamin pyrophosphate in α-keto acid decarboxylations.

There is one obvious virtue of holding on to the flavin coenzyme. In the redox process the flavin changes shape, from planar when oxidized to a butterfly or bent shape when reduced through different oxidation states. If the apoprotein has different affinity for oxidized and reduced forms, this will alter the redox potential of bound flavin and allow the holoenzyme to operate at variable redox potential. For example, D-amino acid oxidase binds FAD with a dissociation constant of 10^{-7} M but binds $FADH_2$ 10 million-fold more tightly, at 10^{-14} M, perturbing the redox potential from −210 mV for the FAD/$FADH_2$ couple free in solution to a value of 0 mV on the enzyme.[1] This, of course, makes the oxidation of D-amino acids to imino acids (the reductive half-reaction) much more favorable. The standard oxidation potential at pH 7, $E^{\circ\prime}$ for $O_2 \rightarrow H_2O_2$, is +300 mV, so the reoxidation of E–$FADH_2$ is also very favorable energetically.

We shall consider flavoprotein reductive and oxidative half-reactions separately below to focus on distinct types of substrate functional groups processed.

The yellow chromophore of the flavin cofactors has been invaluable as an absorbance and fluorescence probe of both the kinetics and the nature of chemical intermediates in both reductive and oxidative half-reactions. Unfortunately, limitations of space preclude proper delineation of such important topics as charge-transfer studies[4] or steady-state and presteady-state kinetic studies which have been so important in enabling dramatic mechanistic progress with flavoenzymes. There are several review and recent conference volumes to set perspective.[5-8]

Flavoprotein Reductants

NADH–NADPH Oxidations. Because NADH and NADPH have more negative redox potentials ($E^{\circ\prime}$ = 320 mV) than most flavoenzymes, these soluble, circulating dihydronicotinamides are important general cellular electron currencies that can be spent to reduce specific flavoproteins. Two examples are adrenodoxin reductase in adrenal cell biosynthesis of steroid hormones such as corticosterone, aldosterone, and various aromatic monooxygenases in bacterial cell metabolism which can function in different specific oxidative half-reactions. It is likely that these half-reactions involve transfer of a hydride equivalent from C_4 of the dihydronicotinamide to N_5 of the flavin.[3] This direct two-electron-transfer step is generally obscured because of the subsequent rapid exchange of N_5–H of enzyme-bound dihydroflavin with solvent protons (eq 6).

$$NAD^3H + \text{E–Fl}_{ox} \xrightarrow{H_2O} NAD^+ + \text{E–FlH}_2 + {}^3H_2O \quad (6)$$

However, there are some enzyme active sites sufficiently sequestered from solvent H_2O that proton exchange is slow and transfer to an exchange-stable product carbon site in the oxidative half-reaction is detectable when [4-^{3}H]NADH is used as reducing substrate.[9,10] Also, the use of synthetic analogues, 5-carba-5-deazaflavin coenzymes,[2,11-15] has allowed direct demonstration of H^- ($^2H^-$, $^3H^-$) transfer to C_5 of the dihydro-5-deazaflavin–enzyme complex with quantitative capture of isotopic label. This is illustrated for an NADH–FMN oxidoreductase from luminescent marine bacteria.[13] Both C_4 of NADH and C_5 of the dihydro-5-deazaflavin are

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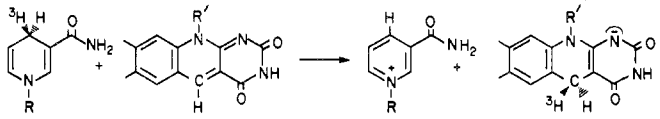
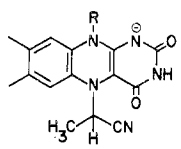
prochiral (i.e., Caabc) centers and flavoproteins show stereospecificity at either prochiral center (eq 7).[15]



(7)

Bacterial flavoenzyme monooxygenases have evolved to utilize the pro-*R* hydrogen[16] at C_4 of dihydronicotinamides,[17] while thiol-reducing, NADPH-oxidizing flavoenzymes such as glutathione reductase, thioredoxin reductase, and dihydrolipoamide dehydrogenase (and also adrenodoxin reductase) are pro-*S* specific.[17,18] The normal oxidized flavin coenzyme has a prochiral trigonal center at N_5, definable as the *re* or *si* face,[16] according to Cahn, Ingold, Prelog nomenclature. Determination of this stereochemistry may in general require high-resolution X-ray structures of flavoenzymes: the recently reported glutathione reductase structure at 3.5 Å suggests the itinerant H^- equivalent from NADPH may attack the flavin *re* face in the enzyme-active site.[19]

Oxidation of Amino Acid and Hydroxy Acid Substrates. The net two-electron oxidation of these functional groups by specific flavoenzymes has been suggested to involve carbanionic transition states or intermediates on the basis of catalytic HX eliminations from such molecules as β-chloroalanine[20,21] and β-chlorolactate,[22] from catalytic oxidation of nitroalkane anions by D-amino acid oxidase,[23] and from several model studies.[24] Carbanion formation as an initial step does not, of course, constitute an oxidation, and the nature of the oxidative step itself (one electron vs. two electron) and the kinetic competence of N_5 adducts are still being debated.[3,24,25] Turnover of nitroethane anion appears to involve an N_5 adduct since it can be trapped by cyanide ion as an inactive aminonitrile (I).[23] More



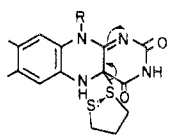
I

physiologically, there is good evidence for a flavin N_5–substrate adduct from collapse of a substrate carbanion onto flavin N_5 during oxidative processing of glycolate by a bacterial lactate oxidase.[26] On the other hand, Bruice and colleagues have adduced evidence in model studies that initial carbanions can be oxidized one electron at a time by radical paths[27] so the issue may still be in doubt especially for such enzymes as monoamine oxidase and glucose oxidase where no structural elements which could help stabilize carbanionic species during oxidation are obviously present.

Oxidation of Dithiols to Disulfides. As noted above, dithiols such as glutathione can be oxidized to the intermolecular disulfide at the expense of NADP reduction to NADPH by the FAD–enzyme glutathione reductase (eq 8).[19] For this reaction type both model

$$2RSH + NADP \xrightleftharpoons{E\text{-}FAD} RSSR + NADPH \quad (8)$$

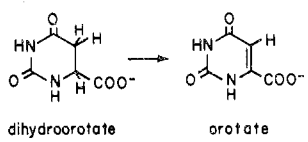
chemistry[28,29] and studies with the mechanistically related dihydrolipoamide dehydrogenase[30] argue convincingly that catalysis proceeds via covalent 4a-flavin–sulfur adducts as indicated, highlighting C_{4a} as an electrophilic center. Attack by the second thiol group on the sulfur of the 4a adduct is favorable because the nascent dihydroflavin acts as a good leaving group (II). The dihydroflavin can then act as a hydride



II

transfer agent in the *second* half-reaction, moving the N_5–H to C_4 of NADP, to regenerate oxidized FAD and yield NADPH.[3]

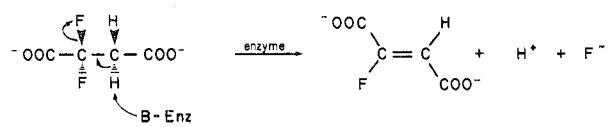
Olefin-Forming Desaturations. The last major dehydrogenation class is exemplified by flavoenzyme-mediated dehydrogenation of succinate to fumarate and butyryl thiolesters to crotonyl thiolesters, or conversion of dihydroorotate to the pyrimidine orotate (eq 9). It



dihydroorotate orotate

(9)

is likely that these desaturations proceed by proton–hydride mechanisms;[3] for example, in butyryl-SCoA dehydrogenation, the C_2 hydrogens are acidic by adjacency to the thiol ester and catalysis is probably initiated by chiral abstraction of a C_2 methylene hydrogen as a proton and elimination of one of the C_3 hydrogens as a hydride ion to flavin N_5 to complete a trans elimination. This is analogous to NADH oxidation. In support of such a proton abstractive initiation, 2,2-difluorosuccinate undergoes catalytic loss of HF by succinate dehydrogenase, yielding fluorofumarate (eq 10).[31]



(10)

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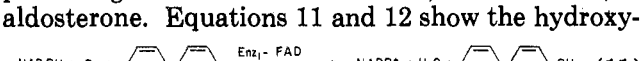
Oxidative Half-Reactions

Since enzymes act catalytically, the above flavin reduction/substrate oxidation reactions also occur in the back direction as dihydroflavin reoxidation/product reduction reactions by microscopically reverse mechanisms, but the predominant directions in the course of metabolism are generally as suggested above.

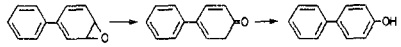
There are, though, two physiologically consequential distinct types of electron acceptors which become reduced in flavin reoxidation half-reactions. These are iron-containing prosthetic groups on the one hand and dioxygen on the other. They will be examined separately.

Iron-Containing Reoxidants. Almost exclusively those flavoproteins that are reoxidized by passage of electrons one at a time to iron centers interact either with non-heme iron–sulfur clusters[32,33] or with heme iron prosthetic groups. These iron redox centers are generally on separate specific protein molecules, but many flavin–iron–sulfur enzymes are known with both redox groups on the same protein, often on distinct polypeptide subunits. A few single polypeptide flavo–heme proteins are known, for example, yeast cytochrome b_2, *E. coli* spermidine dehydrogenase. The iron–sulfur clusters may be 2Fe/2S or 4Fe/4S clusters.[32,33] Both of these clusters and the heme groups also are obligate one-electron acceptors and emphasize the fact that the flavin semiquinone is a key redox intermediate in these electron transfers. In contrast, dihydronicotinamides are not used as direct reductants of biological iron centers probably because the pyridinyl radical is too energetic a species to be kinetically accessible.

The scope of flavin redox interaction with the two types of iron prosthetic groups is encompassed by two variants of multienzyme complexes with NADPH as initial electron donor and a specific heme protein monooxygenase, cytochrome P_{450}, as terminal component.[3] One is a *two-enzyme variant* of flavoenzyme and cytochrome P_{450}, exemplified by the xenobiotic and drug-hydroxylating *detoxification* enzyme systems of liver cells. The other is a *three-enzyme variant* of flavoenzyme, 2Fe/2S cluster protein, and a family of hemeprotein cytochrome P_{450s} specific for particular monooxygenation events in adrenal cell *biosynthetic* processing of cholesterol to cortisone, corticosterone, or aldosterone. Equations 11 and 12 show the hydroxy-



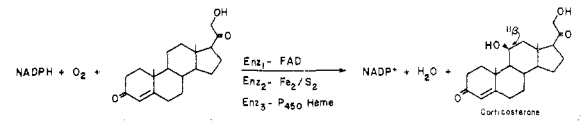
(11)



(12)

lation of biphenyl to 4-hydroxybiphenyl; O_2 is reduced by four electrons and split into H_2O and SOH in the process. NADPH serves as an exogenous donor of two electrons. It reduces the FAD- and FMN-containing E_1 which can then pass electrons one at a time to the hemeprotein monooxygenase.[33a] This is a classic two-electron/one-electron step-down switch.

The three-enzyme variant in eq 13 shows identical stoichiometry and mechanism with the specific intervention of the Fe_2/S_2 adrenodoxin as specific one-electron conduit between reduced flavoprotein and P_{450} sterol 11β-hydroxylase.



(13)

These two multienzyme types are representative of a variety of cellular hydroxylation processes in bacteria, plants, and animals, including sequential vitamin D_3 hydroxylation in liver[34] and kidney,[35] hydroxylation of camphor by pseudomonads,[36] and metabolic activation of polycyclic carcinogens.[37] Their basic strategy is to use the readily available, circulating energy currency, NADPH, as reductant. However, this necessitates a redox switch from the two-electron to the one-electron flavin system before the heme iron oxygenase can reductively activate O_2 for insertion of one oxygen atom into specific cosubstrate.

Reductive Activation of O_2 by Flavoenzyme Monooxygenases. In the above multienzyme monooxygenase complexes, the flavoprotein components have electron transferase activity.[38] On the other hand, neither have they a monooxygenase capacity nor are the reduced forms of those flavoenzymes rapidly reoxidized by O_2. However, there are two separate classes of flavoenzymes which are specifically reoxidized by O_2 physiologically, the oxidases and the monooxygenases.

Flavoprotein oxidases reduce O_2 by two electrons to H_2O_2 in the reoxidative half-reaction (eq 14). Since

$$\text{E–FADH}_2 + O_2 \rightarrow \text{E–FAD} + H_2O_2 \qquad (14)$$

H_2O_2 can be deleteriously reactive, some liver and kidney cells sequester amino acid oxidases and hydroxy acid in specific organelles, called peroxisomes, along with the enzyme catalase which dismutes the H_2O_2 to O_2 and H_2O as it is formed. Monoamine oxidase is a key enzyme in regulating peripheral and central nervous system concentrations of neuroactive amines; it is localized in the inner membrane of mitochondria in cells and is a target enzyme for tranquillizing drugs.

Dihydroflavins, which have a central dihydropyrazine ring, are rapidly autoxidized by O_2 while dihydropyridines are not, possibly because the relevant pathway is by one-electron transfer from the ground state diradical, $^3O_2$. The transfer yields one-electron-reduced O_2, the superoxide anion, and one-electron-oxidized dihydroflavin, the semiquinone radical, as rapidly formed metastable species. There is not universal agreement,[38] appropriately so until direct evidence is available, that this is the sole or major route of O_2 reduction at the enzyme active sites. These radicals may rapidly recombine at the enzyme active site to form a covalent flavin 4a-hydroperoxide which is the common

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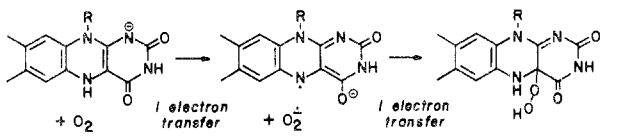
intermediate in both flavoprotein oxidase and monooxygenase chemistry (eq 15). For example, the 4a-peroxide can decompose facilely to yield oxidized flavin and H_2O_2 (eq 16). Typical turnover rates of flavoprotein oxidases are in the range 5–20 nmol s^{-1} $nmol^{-1}$ enzyme, i.e., a complete catalytic event takes 50–200 ms.



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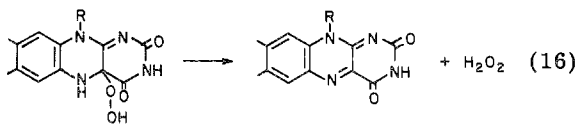
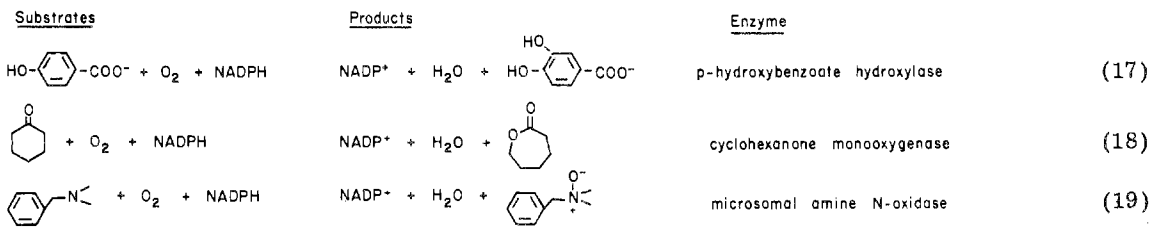


(16)

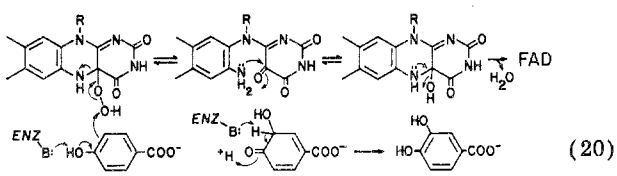
The chemical logic used by flavoprotein *monooxygenases* is reminescent of that in the preceding two sections. Since O_2 undergoes a formal four-electron reduction and cosubstrate oxygenated a formal two-electron oxidation, an additional source of two electrons



is required. The readily available NADH or NADPH is conscripted and used in the reductive half-reaction to generate the O_2-reactive dihydro oxidation state of the flavin. The bound dihydroflavin then reacts with O_2 as pictured in eq 15 to yield a flavin 4a-hydroperoxide. Evidence for this species comes from both rapid low-temperature static studies[39] or kinetic studies[40] showing a kinetically competent reaction intermediate that has essentially the same electronic spectrum (λ_{max} 370 nm (ϵ 8000 M^{-1} cm^{-1}) as authentic N_5-alkylated flavin 4a-peroxides.[41] Particularly convincing are recent model studies by Bruice and colleagues showing that such flavin peroxides are rapid oxygenation reagents for thiols such as thioxane[42] and for tertiary amines to yield amine oxides,[43] mimicking the action of liver flavoprotein N- or S-oxygenases.[44] Whether the flavin 4a-peroxide itself is the actual oxygen transfer agent or progenitor to the actual oxygenating species has been debated by enzymologists and chemists.[24,38,45]

The scope of flavoenzyme-mediated oxygenation chemistry can be examined by category and in comparison to the scope of cytochrome P_{450} mediated oxygenations (vide supra). First, it has been recognized that the heme protein iron oxo species is a relatively more potent oxygenating agent, inserting oxygen into aromatic C–H bonds to give arene oxides (eq 11 and 12) and into aliphatic C–H bonds to give alcohols. Flavoprotein oxygenases apparently cannot oxygenate aromatic rings unless they already have an electron-releasing substituent, such as OH or NH_2. They show no catalytic capacity to work on alkanes.[2,3] Flavin-linked monooxygenases are active for the three reaction types shown in eq 17–19. Cytochrome P_{450} will also carry out cognate oxygenations. To date, the phenolic hydroxylations to catechols and the aldehyde or ketone conversions to acids or esters (apparent Baeyer–Villigers) appear restricted to bacteria; the former especially are key sequences in the biological removal of hundreds of tons of aromatic hydrocarbons from the biosphere every year.[46] The product catechols are next ring opened by iron-dependent dioxygenases, and these sequences represent the major if not the sole source for biodegradation of aromatics.[46] The oxygenation of long-chain aldehydes to acids is catalyzed by flavin-dependent luciferases in luminescent marine bacteria and yields a photon of blue-green light as one of the products.[47]

The reaction catalyzed by *p*-hydroxybenzoate hydroxylase has been examined in detail by Entsch, Ballou, and Massey. They suggest attack of the aromatic π electrons of the *p*-hydroxybenzoate on the distal oxygen of the flavin peroxide. This yields a cyclohexadienone product tautomer ring-opened flavin which would cyclize rapidly back to the 4a-OH pseudobase and then rapidly dehydrate to oxidized flavin and H_2O (eq 20). This and other mechanisms feature



(20)

transfer of an electrophilic or "oxenoid" oxygen atom to electron-rich aromatic substrate. Bruice, in contrast, prefers radical processes again and a superoxide-type combination with substrate radicals.[27]

The chemistry of the aldehyde or ketone monooxygenases appears quite different.[3] In particular, cyclohexanone is oxygenated to ϵ-caprolactone (eq 18) in what may be a Baeyer–Villiger reaction with the 4a-flavin hydroperoxide distal oxygen acting as nucleophile. This is a functional reversal of polarity of the flavin peroxide from the aromatic cases and emphasizes its ambident nature as oxygen-transfer agent.[3,48]

Synthetic and Natural Modified Flavins

Synthetic Flavin Analogues. In the dihydro form of the isoalloxazine ring system the N_5, C_{4a}, C_{10a}, and N_1 positions comprise a diaminoethylene system. Thus, it has been of interest to determine whether nitrogens

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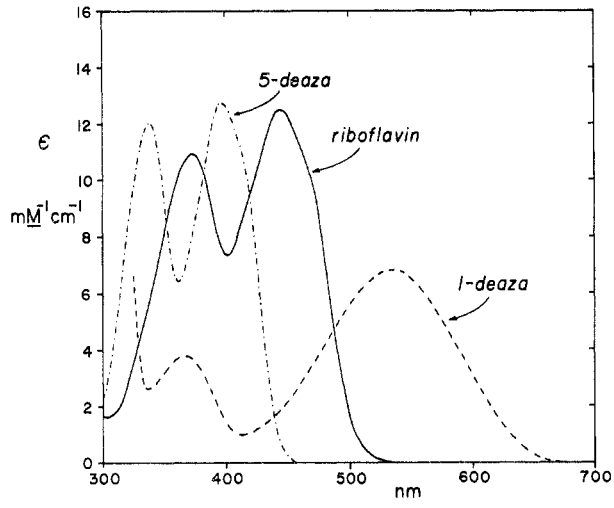
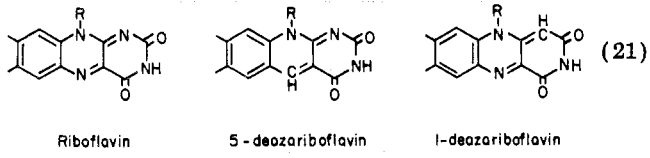


Figure 1.

at both positions 5 and 1 are essential to coenzymatic function (eq 21).[2,48] To that end, syntheses of 5-carba-5-deazariboflavin,[49] 1-carba-1-deazariboflavin,[49] and 1,5-carba-1,5-deazariboflavin[49] have been reported along with enzymic conversion to the FMN and FAD levels.[50] The visible spectra of 5-deaza- and 1-deazariboflavin are compared with riboflavin in Figure 1.



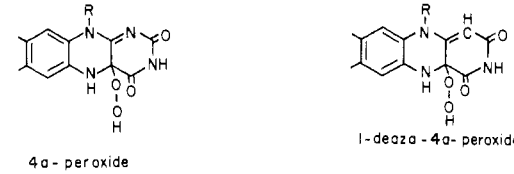
(21)

The 5-deazaflavin system, with central dihydropyridine in the reduced state, is competent for rapid two-electron chemistry only.[2,15,51,52] No catalytic reoxidation by O_2 or by iron cofactors is detectable, and indeed only two-electron reoxidants are active. Thus 5-deazaflavins have only half the redox versatility of flavins and may be "nicotinamides in flavin clothing". Yet, the carbon site at position 5 is inert to rapid exchange with solvent, unlike the parent flavin, and has allowed proof of direct hydride transfer to position 5 from cosubstrates in seven different apoenzymes reconstituted with 5-deazaflavins.[2,15] Substitution of carbon at both positions 5 and 1 leads to a redox inert system in all proteins examined to date.[51]

The 1-deazaflavin system with its deazauracil moiety is more interesting. In contrast to the yellow flavins (and pale yellow 5-deazaflavins), 1-deazaflavins are purple (Figure 1) and, with a redox potential of −290 mV, vs. −210 mV for yellow flavins, the purple flavins are thermodynamically more difficult to reduce. But the central ring is still a pyrazine, and this dominates oxygen reactivity since the dihydro form is reoxidized in milliseconds and a semiquinone is detectable.[51] Thus, 1-deazaflavin-reconstituted enzymes show catalytic competence with D-amino acid oxidase and glucose oxidase[53] and all but one of the dehydrogenases so far examined. The exception is apo yeast cytochrome b_2 which does not even recognize or *bind* 1-dFMN.[54]

Catalytic turnover numbers for the active 1-deazaflavin enzymes vary from ca. 1% to 100% that of native FAD or FMN enzymes, and the V_{max} ratios are diagnostic for whether the redox steps are rate limiting in catalysis.[53]

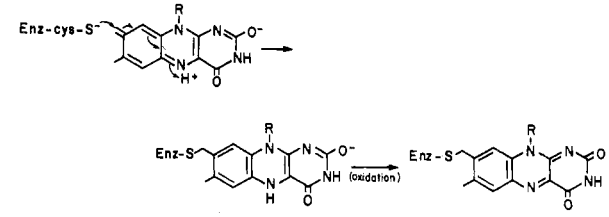
With aromatic ring hydroxylases such as *p*-hydroxybenzoate hydroxylase, 1-deaza-FAD functions to reduce O_2 at 25% the V_{max} of FAD-enzyme, but now all the flux is to H_2O_2.[45] No hydroxylation occurs, and the oxidation of NADPH is completely uncoupled from hydroxylation; the 1dFAD-enzyme is an oxidase—not a hydroxylase. Rapid kinetic studies suggest a 1dFAD-4a-peroxide forms at the active site but breaks down without O–O bond cleavage. In contrast, 1dFAD–cyclohexanone oxygenase is fully coupled for oxygen transfer.[48] This may indicate the 1-deazaflavin 4a-peroxides (eq 22) may be good donors of nucleophilic



(22)

oxygen but not of electrophilic oxygen.

Natural Modified Flavins. While the great majority of flavoenzymes bind FAD or FMN tightly but noncovalently, about a dozen examples are known where the flavin cofactor is covalently attached to an amino acid side chain of the enzyme.[55] In all but one example the linkage is through the 8a-methyl group, to imidazole nitrogens of histidine residues or to a cysteine as a cysteinyl thioether. These linkages might reflect attack by the nucleophilic amino acid side chains on a quinone methide form of the isoalloxazine at C_{8a} (eq 23),



(23)

but in no case is experimental evidence available yet for attachment mechanism. There is one example of covalent attack at C_6, a 6-cysteinylflavin in the bacterial enzyme trimethylamine dehydrogenase.[56] This probably arises by similar nucleophilic attack at C_6 of the benzenoid ring to yield a dihydro species which can be trapped by autoxidation. Covalent attachment of flavin to protein modifies kinetic and thermodynamic features of the flavin which may be essential for the specific reaction in question (e.g., succinate dehydrogenation or amine oxidation).[55] There are reports of both 6-OH-FAD, in glycolate oxidase, and the electron transfer protein of *Megasphera elsdenii*,[57] and 8-OH-FAD, also in the same electron-transfer protein from *M. elsdenii*.[58] But neither appears catalytically active and may represent flavins which have been inactivated by a reaction intermediate such as superoxide. The 6-OH- and, es-

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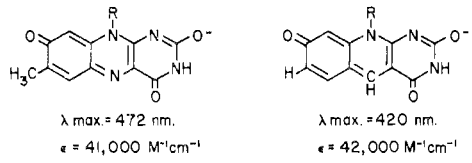
pecially, the 8-OH-FAD have low pK_as for dissociation. Indeed, the pK_a for the 8-hydroxy group is 4.8,[58] while that for the 6-hydroxy group is 7.1.[57] The anionic form is predominantly the *p*-quinone. This is also found with the 8-hydroxy-5-deazaflavin system discussed below (eq 24). Fungi produce a *red* antimetabolite of riboflavin

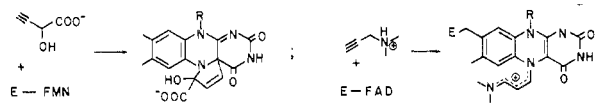


(24)

utilization, roseoflavin,[59] which is the 8-dimethylaminoflavin. It is redox inert in several flavoprotein systems examined to date, possibly because of the altered electronic distribution imposed in the heterocycle, which results from the electron-releasing substituent at the 8 position.[50]

Ghisla and colleagues[57,58] suggested that the 8-hydroxy- and 6-hydroxyflavins might well serve as sensitive indicators of active-site microenvironments. This line has been further developed with 8-thioflavins recently by Massey and co-workers.[60,61] The 8-thioflavins yield three distinct families of chromophores on reconstitution with several apoflavoenzymes, possibly segregating according to sites of protonation and charge stabilization provided by classes of active-site microenvironments.

There are several examples of flavoenzymes inactivated by mechanism-based inactivators or suicide substrates where the flavin coenzyme, not the apoprotein, is modified.[62-64] One case involves the inactivation of monoamine oxidase by drugs containing acetylenic links adjacent to an oxidizable amine function. Dimethylpropynylamine yields an N_5-substituted flavocyanine,[64,65] as do other acetylenic amines (eq 25).



(25)

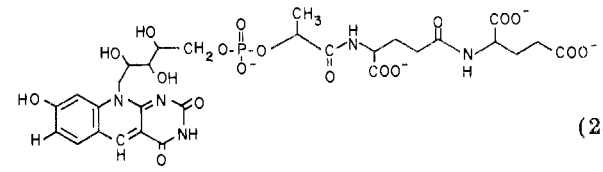
Phenylhydrazine is enzymically processed to phenyldiazene which decomposes with formation of a 4a-phenylflavin derivative.[66] Further evidence that C_{4a} and N_5 are close to the catalytic action is provided by inactivation of L-hydroxy acid oxidase from *Mycobacterium smegmatis* with 2-hydroxy-3-butynoate to yield a 4a,5-cyclic adduct (eq 25).[67,68] When the D-lactate dehydrogenase from *M. elsdenii* processes the D isomer of hydroxybutynoate, a flavin C_6,N_5 adduct is formed.[69] This suggests that the D- and L-enzyme active sites bind the inactivator with opposite orientations at the flavin C_6,N_5,C_{4a} edge. Further, the attack at C_6 probably requires a nucleophilic reagent, suggesting an allenic anion[70] as inactivating agent in all the hydroxybutynoate flavoenzyme derivatizations (eq 26).

(26)

A 5-Deazaflavin Natural Product. The biological occurrence of a natural 5-deazaflavin, in fact an 8-hydroxy-5-deazaflavin, has recently been discovered, by R. Wolfe and his colleagues, as a characteristic metabolite in anerobic methane-producing bacteria. These bacteria, called methanogens, carry out the complex eight-electron reduction of CO_2 to CH_4 at the expense of oxidation of four molecules of H_2 (eq 27). Meth-

$$4H_2 + 4CO_2 \xrightarrow{8e^-} 2H_2O + CH_4 \qquad (27)$$

anogens contain up to 50 mg/kg of cells of a yellow, highly blue fluorescent small molecule with absorbance λ_{max} at 420 nm, factor 420. Factor 420 has been assigned the structure shown in eq 28[71] and that assign-



(28)

FACTOR 420

ment confirmed by the synthesis of authentic 8-hydroxy-7-demethyl-5-deazariboflavin.[72] F_{420} may be the immediate reoxidant for one of the methanogen hydrogenases and the 1,5-dihydro-5-deazaflavin chromophore is the product.[71,72] This dihydro form can then transfer a hydride equivalent to NADP enzymically to yield NADPH. Alternatively, the transfer can be to CO_2 in the presence of formate dehydrogenase to yield formate. The structural alterations at positions 8 and 5 from the riboflavin skeleton alter the reduction potential of F_{420} from the −200-mV midpoint potential value of riboflavin to ca. −340 mV for factor 420.[71] This places it neatly between the $H_2/(2H^+ + 2e^-)$ couple (−420 mV) and the NADP/NADPH couple (−320 mV), enabling F_{420} to function as a redox shuttle between H_2 and NADP, at quite a different redox crossroads than a normal flavin skeleton. Studies are under way to determine the mechanism which may involve a hydride ion and the stereochemistry of redox equivalent transfer between H_2 and factor 420 in methanogens. It is not yet known whether dihydro-F_{420} serves as a mobile reductant directly in the biological production of natural gas.

Conclusion

The flavin tricyclic isoalloxazine skeleton is remarkably well-suited for varied redox chemistry both in having readily accessible one-electron and two-electron reduced states and in possessing multiple sites which can be used for different types of functional group redox transformations. These allow flavin a wide variety of

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redox choices and make it a unique organic molecule in cellular redox metabolism. Recent advances in studies on both synthetic and natural flavin analogues have begun to dissect out features which control sites of hydrogen transfer, control of two-electron vs. one-electron pathways, and modes of O_2 reductive activation as well as to define a new biochemical niche in natural gas production. With the role of flavins in bacterial bioluminescence and plant photoreceptor systems as precedent, we can anticipate that flavins may continue to provide their own spotlight on their roles in redox biochemistry.

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Deciphering the Protein–DNA Recognition Code

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How do proteins interact with specific DNA sequences and as a consequence regulate the expression of genes? Our research is directed toward deciphering this recognition code. The overall objective is to determine why certain DNA sequences are the recognition sites for control proteins. In this way we may be able to learn how to regulate gene expression, to turn genes on, and to turn genes off.

This Account outlines our progress in understanding the recognition or binding of an *E. coli* protein, the *lac* repressor, for its complementary DNA, the *E. coli lac* operator. The binding of *lac* repressor to its operator site on DNA is the biological function of the protein. When the repressor is bound to the DNA, the genes involved in lactose metabolism, the *lac* operon, are not expressed. Conversely when the repressor is not bound to the operator, these genes are expressed.[1] The equilibrium association constant for the binding of repressor protein and operator DNA has been measured and is extremely large (1×10^{13} M^{-1} at $I = 0.05$ M, pH 7.4, 24 °C).[2] In contrast, for *E. coli* DNA deleted for the *lac* operator, the equilibrium association constant for the binding of *lac* repressor has been measured[3] as $(1–3) \times 10^6$ M^{-1}. Thus the binding of *lac* operator for its complementary DNA is highly specific. The objective of our research is to understand how *lac* repressor recognizes *lac* operator, binds specifically to this DNA, and thereby controls the expression of the *lac* operon genes. The approach has involved a series of chemical and enzymatic investigations. Initially *lac* operator DNA was synthesized by a combination of chemical and enzymatic procedures. This DNA was then modified in a sequence-specific manner and tested for altered stability of the *lac* repressor–*lac* operator (RO) complex. In this Account I will review the methodology used for synthesizing various *lac* operators, the results obtained from measuring the stability of these modified *lac* operator–*lac* repressor complexes, and the model for the RO recognition process that was derived from these results.

The basic assumption underlying this research is that the recognition process involves a series of specific hydrophobic and hydrogen bonds between repressor and operator. We have focused our attention on deciphering those functional groups on *lac* operator that are involved in this interaction. The DNA sites are summarized in Figure 1. On a thymine–adenine base pair and in the major groove, the adenine 6-amino group is potentially a hydrogen-bond donor whereas the thymine 4-carbonyl and adenine N7 are potentially hydrogen-bond acceptors. Furthermore the thymine 5-methyl group could interact by hydrophobic bonding. The guanine–cytosine base pair also has hydrogen-bond acceptor groups (guanine N7 and 6-carbonyl) and a hydrogen-bond donor group (cytosine 4-amino) located in the major groove. In the minor groove, both base pairs have hydrogen-bond acceptor groups on the 2-carbonyl (thymine and cytosine) or the 3-nitrogen (adenine and guanine). However guanine is the only base which contains a hydrogen-bond donor located in the minor groove.

Our efforts have been directed toward probing the significance of these various functional groups as potential protein recognition sites. We have altered base pairs by inserting various analogues at specific sites in the *lac* operator sequence and then measured how these alterations affect the repressor–operator interaction. The adenine–thymine base pair has been changed by insertion of bromine or hydrogen for the thymine 5-methyl group. These analogues therefore probe a potential hydrophobic recognition site. The guanine–cytosine base pair has been modified in both major and minor grooves. The cytosine 5-hydrogen has been re-

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