

# Naturally Occurring 5-Deazaflavin Coenzymes: Biological Redox Roles

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Riboflavin (Vitamin B<sub>2</sub>) with its tricyclic isoalloxazine ring is perhaps the most versatile biological redox reagent. In its 5'-phosphate (FMN) and 5'-ADP (FAD) coenzymatic forms, it sits at the crossroads of many enzymic redox routes as a 1e<sup>-</sup>/2e<sup>-</sup> redox switch.<sup>1,2</sup> The ability of riboflavin to interface between obligate two-electron-transfer agents (e.g., NADH) and one-electron-transfer agents, (e.g. iron or molecular oxygen) results from the kinetic and thermodynamic accessibility of the one electron-reduced flavin semiquinone under biological conditions. To dissect out the contributions of the N5-nitrogen (in the pyrazine ring) and the N1-nitrogen (in the uracil ring)<sup>3,4</sup> in controlling the accessibility of the semiquinone form of flavin coenzymes, we have, in collaboration with colleagues at Merck,<sup>5</sup> previously analyzed the redox competence of synthetic riboflavin analogues such as 5-carba-5deazariboflavin (2e<sup>-</sup> steps only), 1-carba-1-deazaflavin 1e<sup>-</sup> and 2e<sup>-</sup> steps), and 1,5-dideazariboflavin (redox incompetent) shown in Table I. The 5-substituent is the crucial determinant for one-electron chemistry since the carbon for nitrogen substitution restricts the 5-deaza system to hydride transfer only. By contrast, the 1-carba analogue converts the uracil ring to deazauracil and makes the tricycle more difficult to reduce but still able to facilitate both 2e<sup>-</sup> and 1e<sup>-</sup> redox reactions.<sup>1,4</sup>

Although 5-deazariboflavin was first synthesized in 1970,<sup>6</sup> this flavin analogue attracted much more of our attention with the report of Eirich in 1978<sup>7</sup> that a 5-deazariboflavin was a naturally occurring structure and in fact was a predominant cellular constituent in anaerobic bacteria that biosynthesize methane. These fastidiously anaerobic methanogenic bacteria are ancient and are thought to be descendants of primordial organisms. They have been designated members of the Archaeobacterial kingdom, a newly defined "third form" of life<sup>8</sup> (vs. eukaryotes and eubacteria). Eirich, Vogels, and Wolfe termed the molecule coenzyme F<sub>420</sub> (Chart I) because of its intense absorbance at 420 nm ( $\epsilon_{420} = 40,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). Coenzyme F<sub>420</sub> is an 8-hydroxy-7-desmethyl-5-deazariboflavin derivative where the 5'-ribityl-OH group is phosphorylated, as in FMN, but is instead in phosphodiester linkage with a lactyl oligoglutamyl grouping (reminiscent of the folate coenzyme oligoglutamyl side chains). The number of glutamates varies among various methanogenic bacteria and also in *Streptomyces* (vide infra). Coenzyme F<sub>420</sub> is a signature molecule for methanogenic bacteria and is present at up to 100 mg/kg of cells.

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**Table I**  
(Carbon) Deaza Analogues of Riboflavin

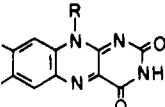
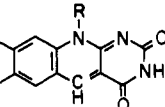
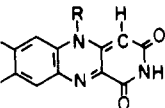
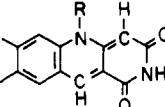
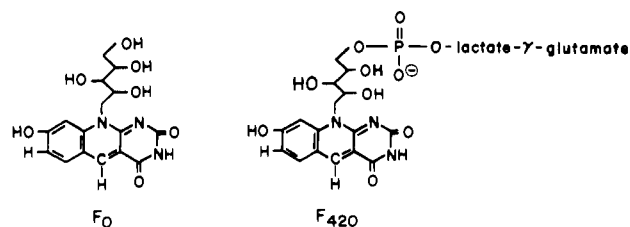
structure	species	E°'	coenzymatic competence
	riboflavin	-210 mV	1e <sup>-</sup> and 2e <sup>-</sup>
	5-deazariboflavin	-310 mV	2e <sup>-</sup> only
	1-deazariboflavin	-280 mV	1e <sup>-</sup> and 2e <sup>-</sup>
	1,5-dideazariboflavin	-370 mV	incompetent

Chart I

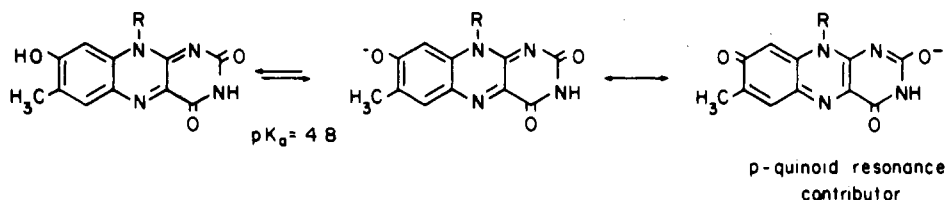


(up to 100mg/kg of  
methanogenic bacteria)

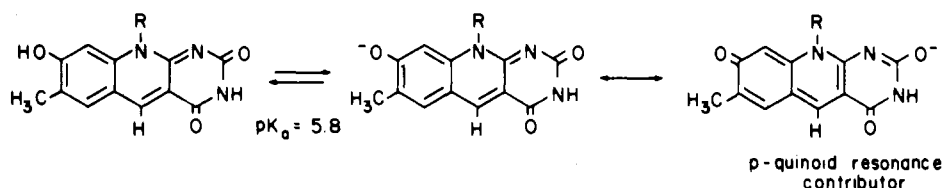
The 8-hydroxy-7-desmethyl-5-deazariboflavin<sup>8</sup> itself, termed F<sub>0</sub>, is excreted into culture medium by some methanogens. The 420-nm chromophore is associated with the 8-hydroxy group and is preceded by chromophoric changes with 8-hydroxyriboflavin where phenol ionization (pK<sub>a</sub> = 4.8) generates a highly colored delocalized anion ( $\lambda_{\text{max}} = 490 \text{ nm}$ ) (Scheme I). The corresponding phenolic pK<sub>a</sub> in synthetic 8-hydroxy-5-deazariboflavin and in F<sub>0</sub> (8-hydroxy-7-desmethyl-5-deaza) is 5.8<sup>7,10</sup> so F<sub>420</sub> is 99% the delocalized chromophoric anion at pH 7.8. On the acid side the 8-

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Scheme I  
8 - Hydroxyriboflavin



8 - Hydroxy - 5 - deazariboflavin



Scheme II

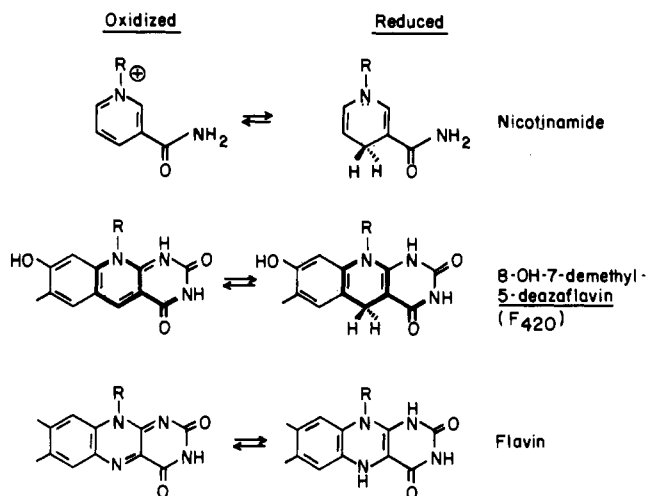
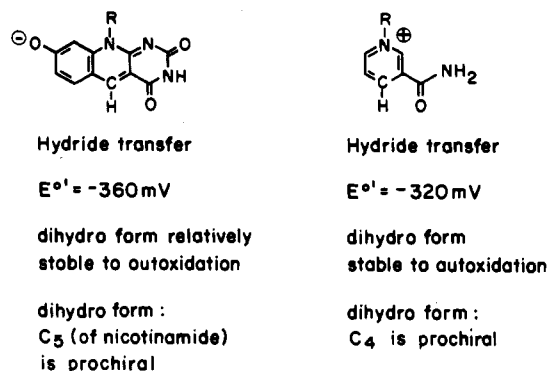
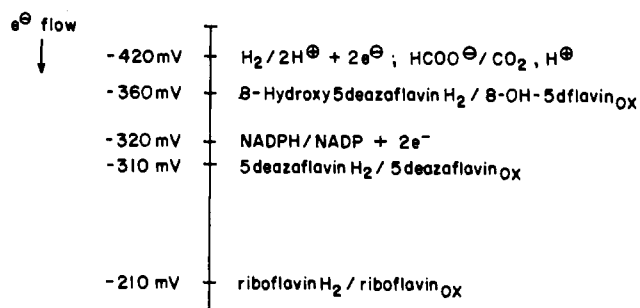


Chart II  
8-OH-5 Deazaflavins vs. NADH



Scheme III



hydroxy-5-deazaflavin system has a  $\lambda_{max}$  of 390 nm with  $E = 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . The electron-rich anionic  $F_{420}$  species is difficult to reduce and  $F_{420}$ -redox enzymes may protonate the *p*-quinonoidal anion at their active sites to facilitate reduction.

Although  $F_{420}$  and  $F_0$  are tricyclic analogues of riboflavin, the 5-carba substitution converts the central pyrazine ring to a pyridine and the structural analogy to NAD-type nicotinamide coenzymes is dramatic as outlined in Scheme II. This pyrazine to pyridine transform (in oxidized state) and corresponding dihydropyrazine to dihydropyridine change in the two-electron-reduced state dominates redox function. The pyridine radicals are much higher in energy than the pyrazine radicals (of flavins), ensuring that both the 8-OH-5-deazaflavins and the nicotinamides are obligate two-electron, hydride-transfer coenzymes in ground-state chemistry.<sup>10,11</sup> It is therefore fruitful to think of  $F_{420}$  as a nicotinamide in flavin's clothing. Thus both  $F_{420}$  and NAD are low-potential, prochiral, air-stable hydride donors in the reduced state (Chart II). Compared to riboflavin, the 5-carba and 8-OH substitutions have ratcheted the redox potential 150 mV more neg-

ative, -210 mV to -360 mV,<sup>7,10</sup> (Scheme III), and so  $F_{420}$  sits at an entirely different redox crossroads from flavin coenzymes. Whereas flavins are reduced by NADH,  $F_{420}H_2$  instead reduces NAD or NADP. In fact with a redox potential of -360 mV, the  $F_{420}/F_{420}H_2$  couple is poised halfway between the  $H_2/2H^+$  (the hydrogen electrode) and  $HCOO^-/CO_2$  couples (-420 mV each) and the NADP/NADPH couple (-320 mV) and this presages the function of three  $F_{420}$ -utilizing methanogen enzymes as discussed below.

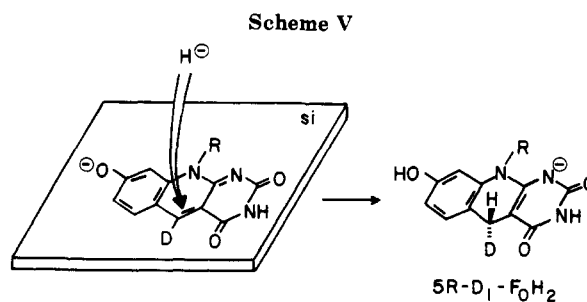
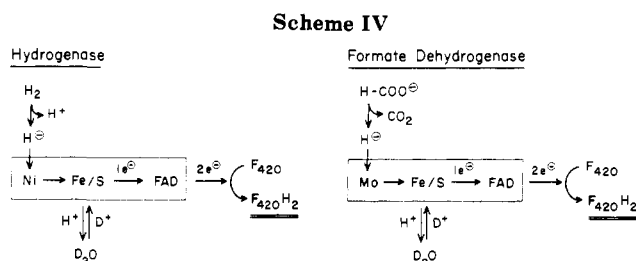
**Redox Roles for Coenzyme  $F_{420}$  in Methanogens**

To set briefly the biological context for function of the low-potential hydride-transfer coenzyme  $F_{420}$  in methanogenic bacteria, we note that methanogens sit at the end of the food chains in the anaerobic digestion of biomass. Methanogenic bacteria can live chemoli-

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Table II  
Coenzyme  $F_{420}$  and Proposed Redox Roles in Metabolism of Methanogenic Bacteria

1. $H_2 + F_{420} \rightleftharpoons F_{420}H_2$	hydrogenase
2. $NADPH + F_{420} \rightleftharpoons NADP + F_{420}N_2$	nicotinamide-deazaflavin transhydrogenase
3. $HCOO^- + F_{420} + H^+ \rightleftharpoons CO_2 + F_{420}H_2$	formate dehydrogenase
4. $CO_2 + \overset{\text{O}}{\parallel} \text{C} \text{SCoA} + F_{420}H_2 \rightleftharpoons \overset{\text{O}}{\parallel} \text{C} \text{COO}^- + \overset{\ominus}{\text{S}} \text{CoA} + F_{420}$	pyruvate synthase
5. $(?) \text{CH}_3 \text{SCoM} + F_{420}H_2 \rightleftharpoons \boxed{\text{CH}_4} + F_{420}$ <i>ATP several proteins</i>	methyl CoM reductase



thoautotrophically on  $CO_2$  and  $H_2$  or they can dismutate acetate to  $CO_2$  and  $CH_4$ . For calibration, about 20% of the flux to make methane is from  $CO_2$  and about 70% from acetate in a municipal biomass waste digester. The estimated annual global amount of methane generated microbially is about  $10^{15}$  g, ( $10^9$  tons), so methane biogenesis is large-scale biochemistry.<sup>12</sup> For the eight-electron reduction of  $CO_2$  to  $CH_4$ , 4  $H_2$  molecules are oxidized by action of hydrogenase enzymes. The methanogenic bacteria contain at least six novel coenzymes, several involved in the  $CO_2 \rightarrow CH_4$  pathway (e.g. methanofuran,<sup>13</sup> methanopterin,<sup>14</sup> methyl CoM,<sup>15</sup> the nickel-containing tetrapyrrolic coenzyme  $F_{430}$ <sup>16,17,18</sup> and three novel nickel-containing enzymes). The nickel is tightly and stoichiometrically bound in each enzyme. One such enzyme is the  $F_{420}$ -reducing hydrogenase,<sup>19</sup> the second a methyl-*S*-coenzyme *M* reductase,<sup>20</sup> the third a carbon monoxide dehydrogenase involved in acetate synthesis.<sup>21</sup> Intense efforts are underway to characterize ligand structure and nickel redox function in these three enzymes which seem to be a nickel hydrogenation catalyst, nickel desulfurization catalyst, and nickel biocarbonylation catalyst, respectively.

Five proposed roles for  $F_{420}$  in methanogen metabolism are summarized in Table II. The  $F_{420}$ -reducing hydrogenase,<sup>19,23</sup> the  $F_{420}$ -reducing formate de-

hydrogenase,<sup>24</sup> and the NADP- $F_{420}$  oxidoreductase<sup>25</sup> have been purified to homogeneity while purported roles for  $F_{420}H_2$  in driving reductive carboxylation of acetyl CoA<sup>26</sup> and of methyl thioether cleavage to methane<sup>20</sup> have only been demonstrated in crude cell-free extracts.

The simplest enzyme, the transhydrogenase, catalyzes a direct hydrogen transfer from one of the  $C_5$  prochiral methylene hydrogens of the dihydro form of  $F_{420}$  directly to  $C_4$  of NADP in an apparent hydride transfer.<sup>11</sup> By contrast  $D_2$  and  $DCOO^-$  do not yield 5-deuterio dihydro  $F_{420}$  in hydrogenase<sup>27</sup> or FDH-mediated reactions (see Scheme IV).<sup>28</sup> The itinerant hydride (deuteride) equivalent exchanges with solvent protons at some stage in catalysis as demonstrated by production of chiral 5- $D_1$ - $F_0H_2$  species in  $D_2O$ . The absolute chirality of these enzymically generated monodeutero dihydrodeazaflavins has recently been established by Yamazaki et al<sup>29,30</sup> by degradation to hydroxyethyl-quinolineone and ORD comparison to authentic deuterated samples. For all three  $F_{420}$ -utilizing enzymes the incoming hydride equivalent is added to the Si face at the  $C_5$  locus of the 8-hydroxy-5-deazaflavin substrate as depicted in Scheme V.

The  $F_{420}$ -reducing hydrogenase and formate dehydrogenase each have three subunits and three associated redox centers; the  $H_2$ ase<sup>23</sup> has nickel, iron-sulfur clusters, and FAD while the FDH<sup>31</sup> has molybdenum, iron-sulfur clusters, and FAD (Scheme IV). It is likely that the nickel site and the molybdenum site respectively are initial sites for *hydride transfer in* from  $H_2$

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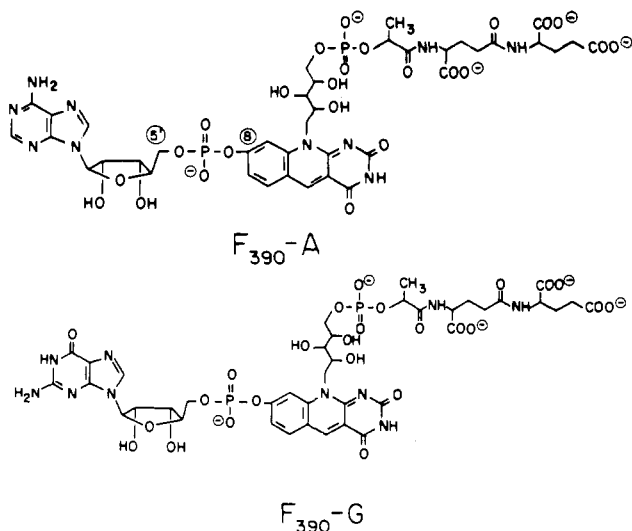
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Chart III  
Structure of Methanogen  $F_{390}$ 's



or from  $HCOO^-$ . The Fe/S centers are likely one-electron conduits to the bound FAD from which, at the  $FADH_2$  level, *hydride transfer out* occurs to  $C_5$  of  $F_{420}$ . The bound FAD in each enzyme is the required  $1e^-/2e^-$  redox switch, interfacing between the one electron Fe/S clusters and the two electron-accepting 8-hydroxy-5-deazaflavin substrate. The net hydride-transfer process is obscured in both hydrogenase and formate dehydrogenase catalysis by proton exchange with solvent which could occur at any of the three redox sites in either enzyme, for example from N5 of bound  $FAD_2$  as one case. Indeed, it is known that intact methanogenic bacteria growing on  $CO_2$  and  $D_2$  in  $H_2O$  generate  $CH_4$  almost quantitatively,<sup>32</sup> consistent with the above data on the hydrogenase. Hydrogenase from which FAD has been removed can still reduce one-electron acceptors (methyl viologen) but not  $F_{420}$  until FAD has been added back.<sup>24</sup> The FAD bound in hydrogenase and formate dehydrogenase must have oxidized FAD bound more tightly than  $FADH_2$ , to lower bound FAD redox potential from  $-210$  mV towards the  $F_{420}/F_{420}H_2$  couple ( $-360$  mV). This is precented in flavoproteins such as flavodoxins, where the one-electron flavin potential is ca.  $-400$  mV.

In oxidizing  $H_2$  or  $HCOO^-$  and passing on the two electron equivalents to generate  $F_{420}H_2$ , methanogenic bacteria generate a kinetically stable, diffusible, low potential energy currency to spend in driving cellular metabolism. By passing electrons to  $F_{420}$  ( $-360$  mV) rather than to NADP ( $-320$  mV) directly, the bacteria harvest an additional 1.6 kcal/mol in the energy available in  $H_2$  or  $HCOO^-$  oxidation and so make a better living with this finely tuned 8-hydroxy-5-deazaflavin energy currency.

### $F_{420}$ in an Oxidant Stress Response?

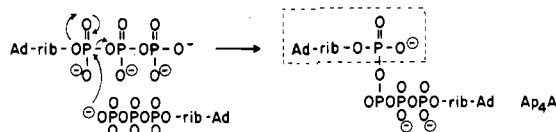
When the fastidiously anaerobic methanogenic bacteria are exposed to  $O_2$ , they are rapidly inactivated. On exposure of intact cells (but not cell-free extracts) to air, the  $F_{420}$  content (as high as 100 mg/kg) of the cells rapidly declines.<sup>33</sup> While this was thought initially to

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(33) Schonheit, P.; Keweloh, H.; Thauer, R. *FEMS Microbiol. Lett.* 1981, 12, 347.

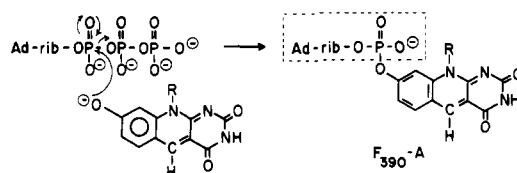
Scheme VI

### 1. Adenylyl Transfer to ATP in Synthesis of Oxidant Stress Alarmone AppppA (Bruce Ames et al)



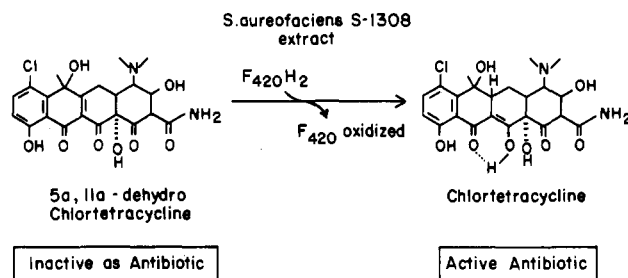
Catalyzed by various bacterial aminoacyl t-RNA synthetases

### 2. Proposed Adenylyl Transfer in Conversion of $F_{420} \rightarrow F_{390-A}$ Under Oxidant Stress



Scheme VII

### The Last Step in Chlortetracycline Biosynthesis in *Streptomyces Aureofaciens*



$F_{420}H_2$  is the specific reductant  
(initially termed "cosynthase I")

be some  $F_{420}$  destructive event, we have shown that as  $F_{420}$  declines two reciprocally accumulating  $F_{390}$  chromophores are the 8-O-AMP and 8-O-GMP esters of  $F_{420}$ <sup>34</sup> (Chart III). These adenylylated and guanylylated  $F_{420}$  molecules are not hydrogenase substrates and may signal the cells to shut down energy metabolism in response to oxidative stress. An analogy in these Archaeobacteria may be derived to recent work by Ames and colleagues in eubacteria subjected to oxidant stress.<sup>35</sup> *Salmonella* rapidly synthesize Ap4A, possibly as an "alarmone" regulatory signal to selectively shut down certain metabolic circuits (Scheme VI, eq 1). Ap4A is generated by adenylyl (AMP fragment) transfer from one ATP to a second ATP in the active site of an aminoacyl transferase. The adenylylation of the phenoxide nucleophile of  $F_{420}$  could be a corresponding Archaeobacterial alarmone response in  $F_{390A}$  generation (Scheme VI, eq. 2).  $F_{390A}$  may also be a primordial variant of an AMP-O-tyrosyl regulatory strategy seen in eubacterial and eukaryotic metabolism.

### $F_{420}$ Redox Roles in Non-Methanogenic Organisms

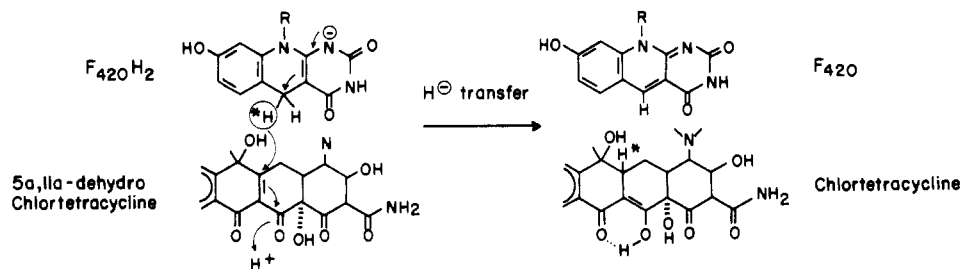
While one could perhaps initially regard 8-hydroxy-5-deazaflavins as an evolutionary cul de sac in coenzyme evolutionary development adapted for the low potential metabolism of methanogen life, it turns out that  $F_{420}$  has also been detected in *Streptomyces*,<sup>36</sup> in mycobacteria,<sup>37</sup> in halobacteria,<sup>38</sup> and in the blue-green alga

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**Scheme VIII**  
Possible Mechanism for Chlortetracycline Formation:  
 $F_{420}H_2$  as a Hydride Donor for Conjugate Addition



*Anacystis nidulans*<sup>36</sup> in the 7 years since its initial identification.

Two remarkable and distinct coenzymatic roles have been documented to date in *Streptomyces* metabolism and are summarized below.

**$F_{420}$  as Redox Coenzyme in the Last Step of Tetracycline Biosynthesis**

In 1960 McCormick and colleagues at Lederle,<sup>39</sup> working on the biosynthesis of the antibiotic chlortetracycline in blocked mutant strains of *Streptomyces aureofaciens*, showed that conversion of the penultimate metabolite 5a,11a-dehydrochlortetracycline (inactive as an antibacterial) to the active chlortetracycline (Scheme VII) required a small diffusible molecule they termed "cosynthase I". They reported a UV-visible spectrum (420 nm chromophore) of cosynthase I and an elemental analysis (that was not quite correct) and did not identify the then unknown structure. Subsequent to the initial studies reported on  $F_{420}$  and  $F_0$ <sup>7,9</sup>, we gave the Lederle group a synthetic sample of  $F_0$ , and they confirmed that cosynthase I was a triglutamyl  $F_{420}$  species.<sup>40</sup> While specific studies with 5-<sup>[3H]</sup>- $F_{420}H_2$  remain to be done it is quite likely that dihydro  $F_{420}$  is acting as a specific hydride-transfer donor in 1,4-addition to the enone system in enzymic conversion of inactive precursor to active chlortetracycline as shown in Scheme VIII. There is indirect evidence that  $F_{420}H_2$  fulfills the same role in the last step of oxytetracycline biosynthesis<sup>41</sup> in other strains of *Streptomyces* and these may be the organisms of choice for analysis of the pathway of  $F_{420}$  biosynthesis since those genes may be clustered in these bacteria and amenable to cloning and overproduction.

**$F_{420}$  as Coenzyme in Enzymic Photoreversion of *cis-syn*-Cyclobutane Dimers in UV-Damaged DNA**

The most prevalent covalent change in DNA after absorption of UV light is the formation of cyclobutane rings as two adjacent intrachain thymine residues photodimerize to *cis-syn*-thymine dimers ( $T \langle \rangle T$ ) (Scheme IX). Enzymic repair is crucial for organism survival and maintenance of DNA information content, and repair occurs both in dark and light-mediated processes. The dark reaction excision-repair enzyme

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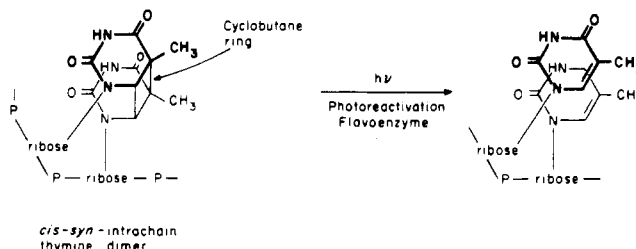
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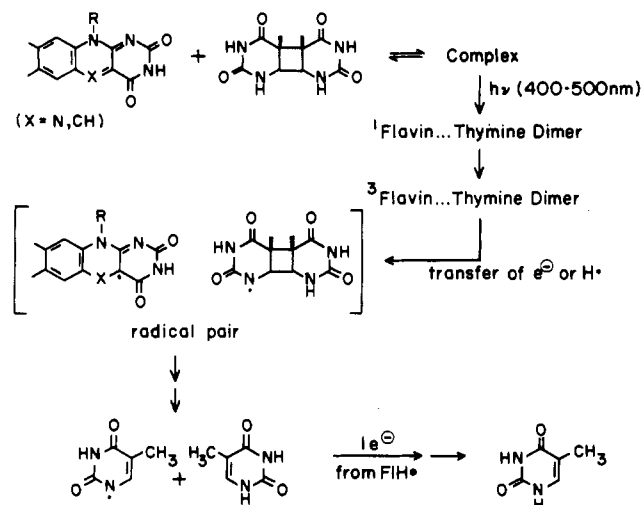
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**Scheme IX**



**Scheme X**  
Possible Mechanism for Flavins, 5-Deazaflavins as Sensitizers for Dimer Photomerization



systems are common and efficient for many types of DNA adducts,<sup>42</sup> including  $T \langle \rangle T$  repair, while photoreversion is essentially limited to  $T \langle \rangle T$  repair. Photoreversion enzymes use visible light, at wavelengths of 360–440 nm where nucleic acids do not absorb, to drive the net retro 2 + 2 cycloreversion of the cyclobutane-containing  $T \langle \rangle T$  dimers in an apparent photosensitized process. Two types of photoreactivation enzymes have been purified recently. Enzymes from *E. coli*<sup>43,44</sup> and yeast<sup>45</sup> contain FMN while that from *Streptomyces griseus*<sup>46</sup> has stoichiometrically bound coenzyme  $F_{420}$  and has a photoactivation spectrum at 420 nm as does the photoreaction enzyme from the blue green alga *Anacystis nidulans*. Eker noted 7% nonenzymic photomonomerization of  $T \langle \rangle T$  by  $F_0$ , and we have recently shown several flavins and 5-deazaflavin can act, in the

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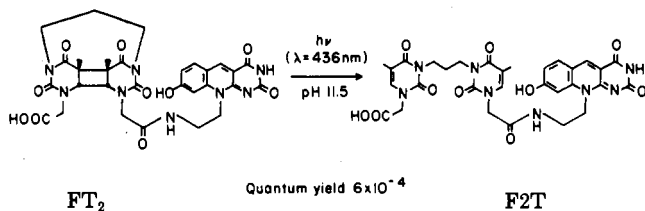
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**Scheme XI**  
**Intramolecular Photosynthesized Cleavage by**  
**8-Hydroxy-5-Deazaflavin of a Covalently Tethered**  
***cis-syn*-Thymine Dimer Is Successful**



absence of enzyme, as catalytic photosensitizers of the cycloreversion process.<sup>47</sup>

In contrast to ground-state chemistry, in the excited state both the flavin and 5-deazaflavins have accessible semiquinone states and 5-deazariboflavin is a broadly useful, low-potential, one-electron photoreductant (e.g., for difficultly reducible redox proteins).<sup>48</sup> A possible route for flavin or 5-deazaflavin photosensitized reversion of  $T \leftrightarrow T$  is indicated in Scheme X, postulating one-electron transfer from  $T \leftrightarrow T$  dimer to yield the thymine radical cation and (deaza)flavin semiquinone complex. Radical fragmentation and back electron transfer would complete photomonomerization.<sup>47</sup> Although our model system was successful with 5-deazariboflavin and 8-methoxy-5-deazariboflavin, we had no success in irradiation with the natural 8-hydroxy-5-deazaflavin chromophore in intermolecular photosensitizations—possibly due to the rapid, competing photodestruction of  $F_0$ . We have recently prepared an intramolecularly tethered *cis-syn*-thymine dimer-8-hydroxy-5-deazaflavin species<sup>49</sup> shown in Scheme XI. On irradiation of the dimeric  $FT_2$  at the deazaflavin  $\lambda_{max}$  (426 nm),  $T \leftrightarrow T$  intramolecular monomerization, to the cleaved, rearomatized  $F_2T$ , is achieved albeit at low quantum yield ( $6 \times 10^{-4}$ ). These experiments begin to map out the role of the excited

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state of the 8-OH-5-deazaflavin coenzymes in the biologically crucial and chemically interesting retro 2 + 2 repair of thymine dimers in DNA.

### Concluding Remarks

The vitamin B<sub>2</sub>-based flavin coenzymes have been known and studied for over 50 years and have been found to be among the most versatile biological redox catalysts, for electron transfers, for substrate dehydrogenations and for reductive activations of dioxygen. The natural 5-deazaflavins have been identified only since 1978 but have already been detected in some unusual biological redox niches. The tricyclic 5-deazaalloxazine nucleus is a functional hybrid (and perhaps an evolutionary one) between monocyclic nicotinamide and tricyclic flavin coenzymes. In the ground state the 8-hydroxy-5-deazaflavin coenzymes appear to be low potential variants of the nicotinamide-type hydride transfer coenzyme. They are perfectly suited to harvest energy from gaseous H<sub>2</sub> in hydrogenase catalysis. As a nicotinamide mimic  $F_{420}$  allows detection of direct hydrogen transfer and stereochemical outcome at the prochiral C<sub>5</sub> methylene locus in the dihydro oxidation state. On photo excitation, the  $\lambda_{max}$  is in the blue-green region of the visible spectrum, the one-electron-reduced deazaflavin semiquinone becomes kinetically accessible and is a stronger one-electron-reducing agent than the corresponding flavin semiquinone. This property is well-tailored to function as photosensitizing coenzyme to initiate a radical pathway for cleavage of the cyclobutane-containing thymine dimers in UV-damaged DNA by the photoreversion enzyme, DNA photolyase. The 5-deazaflavin structure may have been an early experiment in redox coenzyme evolution to yield a molecule with properties hybrid between nicotinamide and flavin redox coenzymes.

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Registry No. Coenzyme  $F_{420}$ , 64885-97-8.