state of the allyl system as well as of the orthogonal transition state is assumed. The orthogonal transition state consists of a localized radical and therefore the cyano groups should influence its energy to a higher extent than the delocalized ground state.

Presently we are trying to extend our analysis of rotational barriers in allyl systems.

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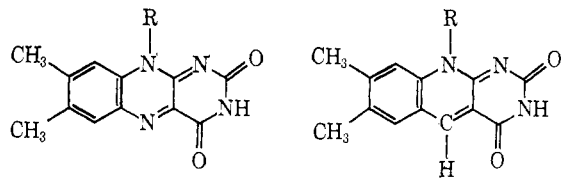
Enzymatic Reduction of 5-Deazariboflavine from Reduced Nicotinamide Adenine Dinucleotide by Direct Hydrogen Transfer

Sir:

Elucidation of the chemical role of the flavine coenzyme remains a compelling question in flavoenzyme oxidative catalysis.[1] Recent studies have suggested proton abstraction and discrete two-electron transfer during enzymatic oxidation of α-hydroxy acids and α-amino acids.[2] Model studies of Bruice and Brüstlein[3] on nicotinamide oxidations have been invoked for hydride transfer in flavoenzyme oxidations of pyridine nucleotides.[4] The ideas of molecular complex formation preceding oxidation[5] and the possibility of covalent adducts between substrates and flavine coenzyme have been recently advanced.[6] With regard to covalent catalysis, Hamilton[6] has favored nucleophilic attack at C_{4a}; others have pointed to N_5 as an electrophilic center on the basis of its reactivity with sulfite,[7] and on theoretical grounds.[8]

The use of 5-deaza analogs (II) of flavine coenzymes (I), R = ribityl for riboflavine (RF) and deazariboflavine (dRF) and R = ribityl-5′P for FMN and dFMN,



I

II

in which a carbon atom replaces nitrogen at position 5 may allow distinction of some of these possibilities especially if they serve as coenzymes. Tollin and colleagues[9] have observed 5-deazaFMN binds to an apoflavoprotein while Bruice, *et al.*, have demonstrated direct hydrogen transfer in model systems between deazaisoalloxazines and either nicotinamides[3] or pyridoxine.[10]

In this communication we report enzymatic evidence that 5-deazariboflavine functions coenzymatically, *undergoing reduction by direct hydrogen transfer* from NADH. We have employed partially purified NADH:FMN oxidoreductase[11] (specific activity = 7.1 U/mg; molecular weight = 24,000 daltons) from *Beneckea harveyi* (formerly *Photobacterium fischeri* strain MAV) which catalyzes reaction 1; unlike most flavoenzymes,

$$\text{NADH} + \text{FMN} + \text{H}^+ \rightleftarrows \text{NAD}^+ + \text{FMNH}_2 \qquad (1)$$

this reduces FMN as a substrate rather than as a tightly bound coenzyme, with the product $FMNH_2$ feeding into the luciferase reaction.[12] We have found that riboflavine can replace FMN in this enzymatic reaction with a K_m of 8.2×10^{-7} M and an equivalent V_{max}. Preliminary tests showed 5-deazariboflavine[13] to be a competitive inhibitor ($K_i = 8. \times 10^{6}$ M) of riboflavine reduction. We then tested the synthetic deazariboflavine, λ_{max} 338 (ϵ 11,000) and 396 nm (ϵ 12,000),[13] for enzyme-catalyzed reduction. Since reduced deazariboflavine reoxidized some 10^4-fold more slowly in air than riboflavine itself ($t_{1/2}$ = 180 min *vs.* <1 sec),[9] it was feasible to monitor loss of absorbance at 396 nm as a direct measure of reaction. We consistently see enzyme-catalyzed reduction of dRF concomitant with NADH oxidation.[14] The rate is 0.3% of the rate observed with riboflavine itself under the given conditions. For an as yet undetermined reason, incomplete reduction of added dRF occurs (*e.g.*, with 1.5 A_{396} units, reduction levels off at about 50% reduction). The nature of this gradual inhibition is apparently not simple accumulation of reduced dRF nor the equlibrium position of the reaction and is under investigation.

As a prelude to testing for enzymatic direct transfer of hydrogen from C_4 of NADH to C_5 of the dRF during reduction, we determined the chirality of transfer to riboflavine itself by measuring ^3H released into water from either 4-(*R*)-[^{3}H]NADH or 4-(*S*)-[^{3}H]NADH.[15]

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(14) Incubation conditions were, in a final volume of 1.0 ml of 0.050 M NaPPi pH 8.6, 125 nmol of dRF, 95 nmol of NADH, and 1.5 units of enzyme. When RF was assayed, 175 nmol of NADH were mixed with 33 nmol of RF.

(15) The 4-(*S*)-[^{3}H]NADH was prepared from 4-[^{3}H]NAD (Amersham–Searle) by reduction with ethanol and yeast alcohol dehydrogenase and the 4-(*R*)-[^{3}H]NADH by incubation of glyceraldehyde 3-phosphate and glyceraldehyde 3-phosphate dehydrogenase in the presence of arsenate.

The enzyme shows clear preference for ^3H transfer from the *R* position[16] but, on the surface, would appear not totally stereospecific as evinced by $^1/_8$th as much ^3H transfer from the *S* position. This unlikely result may have precedence with the flavoenzyme orcinol hydroxylase.[17]

Repetition of these experiments with dRF confirms the same stereoselectivity (but not complete stereospecificity) for *R*-^3H transfer from C_4 of NAD^3H now into a stable nonexchangable locus (presumably C_5) in reduced and subsequently air reoxidized dRF. In one experiment, the reisolated [^3H]dRF had a sixfold higher specific radioactivity from (*R*)-[^3H]NADH than from (*S*)-[^3H]NADH. In the experiment shown in Table I a fourfold differential occurred. The control incubation without enzyme demonstrates that both dRF reduction and tritium transfer are enzyme catalyzed. The [^3H]-dRF from the enzymatic incubations was purified with removal of radioactive pyridine nucleotides by batch treatment with DEAE-cellulose. The uv spectrum of the supernatant indicated presence of partially reduced dRF.

Table I. Enzymatic Tritium Transfer to Riboflavine and Deazariboflavine

Experiment	4-[^3H]-NADH[a]	Flavine	Enzyme	Product formation[b] (nm)	^3H_2O[c] (μCi/mol)	[^3H]dRF[d] (nCi/nmol)
1	4-*R*	RF	+	175	8	
2	4-*S*	RF	+	175	1	
3	4-*R*	dRF	+	31		1.40
4	4-*S*	dRF	+	56		0.34
5	4-*R*	dRF	−	0		0.05

[a] Incubations were as described for nonradioactive experiments.[14] The specific radioactivity of commercial 4-[^3H]NAD was 10 nCi/nmol. This was reduced enzymatically to the two chiral 4-[^3H]-NADH species. [b] With RF, product formation was measured by NADH oxidation at 340 nm; with dRF direct reduction of the deazaisoalloxazine chromophore was followed at 396 nm. [c] Measured as ^3H rendered volatile during enzymatic incubation and lyophilization. Values are corrected for a small nonenzymatic blank. Without any kinetic isotope selection,[16] a specific activity of 31.5 μCi/mol of ^3H_2O would be expected. [d] These values represent specific activity of isolated [^3H]dRF corrected for the different amounts of dRF reduced in experiments 3 and 4 but not for ^3H lost in nonenzymatic reoxidation of reduced [^3H]dRF included as part of the isolation.

The [^3H]dRF after air oxidation was passed through a 1 × 85 cm column of Biogel P_2 in 0.1 *M* NH_4HCO_3 and the fluorescent deazaflavine band was collected, lyophilized, redissolved in H_2O, and analyzed on silica gel tlc (as a single fluorescent spot). As a final identification, the [^3H]dRF samples were phosphorylated with partially purified riboflavine kinase[18] to form [^3H]dFMN cleanly separable from unreacted [^3H]dRF on silica gel tlc.[19]

These results demonstrate coenzymatic function for 5-deazariboflavine in an enzymatic oxidation, proving stereoselective, direct hydrogen transfer from NADH and establishing the biological relevance of the model system of Bruice and coworkers.[3] Observed direct hydrogen transfer is consistent with a hydride ion transfer in this enzymatic oxidation and further indicates the N_5 is not a unique electronic determinant for coenzymatic function, supporting the calculations of Song.[8] Given the greater resistance of reduced deazaflavines to air oxidation,[9] it may prove possible to use the reduced forms of the deazaflavines, chiral at carbon 5,[20] to probe stereochemistry of flavoenzyme reactions in a way not possible with the flavine coenzymes themselves.

Simultaneously with this work, Hersh, *et al.*, have found that dFMN functions coenzymatically with amino acid substrates for a bacterial *N*-methyl glutamate synthetase.[21] This parallels our finding that *M. smegmatis* apolactic oxidase reconstituted with dFMN undergoes enzyme-catalyzed reduction by L-lactate.[22]

Acknowledgments. We wish to gratefully acknowledge the valuable assistance of Drs. J. Becvar and J. W. Hastings for provision of crude extracts of the reductase and assistance in the enzyme purification. This research was supported by National Institutes of Health Grant No. 20011.

(16) We have not yet quantitated the kinetic isotope effects, implicit in Table I, by careful rate measurements.

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(19) Brickman plastic-backed plates, H_2O as solvent, [^3H]dFMN had a mobility of 0.6, [^3H]dRF a mobility of 0.2.

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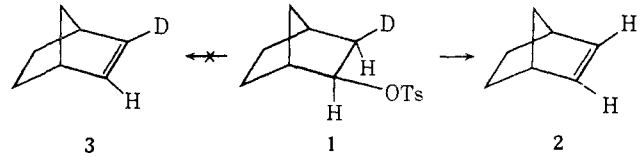
Stereochemistry in β Eliminations from *exo*-2-Norbornyl Tosylate. The Effect of Base Association

Sir:

In 1970, Brown and Liu[1] reported that eliminations from *exo*-2-norbornyl-*exo*-3-*d* tosylate, **1**, induced by the sodium salt of 2-cyclohexylcyclohexanol in triglyme produced norbornene, **2**, but no 2-deuterionorbornene, **3**. The observed exclusive exo–syn elimination stereochemistry was consistent with previous investigations of substituted norbornane reactions in which favoring of syn–exo elimination over anti–endo-H elimination by a factor of 100 or greater has been noted.[2]



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