Enzyme-Catalyzed Redox Reactions with the Flavin Analogues 5-Deazariboflavin, 5-Deazariboflavin 5′-Phosphate, and 5-Deazariboflavin 5′-Diphosphate, 5′→5′-Adenosine Ester

Jed Fisher, Rob Spencer, and Christopher Walsh*

ABSTRACT: The ability of 5-deazaisoalloxazines to substitute for the isoalloxazine (flavin) coenzyme has been examined with several flavoenzymes. Without exception, the deazaflavin is recognized at the active site and undergoes a redox change in the presence of the specific enzyme substrate. Thus, deazariboflavin is reduced catalytically by NADH in the presence of the *Beneckea harveyi* NAD(P)H:(flavin) oxidoreductase, the reaction proceeding to an equilibrium with an equilibrium constant near unity. This implies an E_0 of −0.310 V for the deazariboflavin–dihydrodeazariboflavin couple, much lower than that for isoalloxazines. With this enzyme, both riboflavin and deazariboflavin show the same stereospecificity with respect to the pyridine nucleotide, and despite a large difference in V_{max} for the two, both have the same rate-determining step (hydrogen transfer). Direct transfer of the hydrogen is seen between the nicotinamide and deazariboflavin in both reaction directions. DeazaFMN reconstituted yeast NADPH:(acceptor) oxidoreductase (Old Yellow Enzyme), and deazaFAD reconstituted D-amino acid:O_2 oxidoreductase and *Aspergillus niger* D-glucose:O_2 oxidoreductase are all reduced by substrate at approximately 10^{-5} the rate of holoenzyme; none are reoxidized by oxygen or any of the tested artificial electron acceptors, though deazaFADH$^-$ bound to D-amino acid:O_2 oxidoreductase is rapidly oxidized by the imino acid product. Direct hydrogen transfer from substrate to deazaflavin has been demonstrated for both deazaFAD-reconstituted oxidases. These data implicate deazaflavins as a unique probe of flavin catalysis, in that any mechanism for the flavin catalysis must account for the deazaflavin reactivity as well.

In the study of flavoenzyme-mediated oxidative catalysis, elucidation of how electrons are transferred into the flavin coenzyme from substrate and then back out to acceptor (coenzyme reoxidation) remains a central and unresolved question. One key locus of reactivity in the isoalloxazine ring of flavins has been postulated to be nitrogen 5, the unsubstituted nitrogen of the central ring, implicated as a possible site of entry of substrate-derived electrons and as an electrophilic center subject to attack by nucleophiles for covalent adduct formation. With the synthesis of the 5-deazaisoalloxazines, carbon replacing nitrogen at position 5, by Cheng and his colleagues in 1970 (O'Brien et al., 1970) it became possible in principle to evaluate the ability of deazaflavins to serve as oxidative cofactors. In the accompanying paper we have detailed methods for enzymatic synthesis of 5-deazaFMN[1] and 5-deazaFAD, and examined some of the chemistry that would be relevant to oxidative enzymatic catalysis (Spencer et al., 1976).

In this paper we describe mechanistic studies with a number of flavoenzymes which can reduce and oxidize the deazaflavins at the level of deazariboflavin, deazaFMN, or deazaFAD. With the NADH:flavin oxidoreductase from the bacterium *B. harveyi* and the NADPH:O_2 oxidoreductase (Old Yellow Enzyme) we have examined representatives of the nicotinamide-oxidizing flavoenzymes. With D-amino-acid oxidase from pig kidney and glucose oxidase from *Aspergillus niger* we have examined the ability of deazaFAD to react with a different class of substrates, those which generate carbonyl-containing products. We have reported some preliminary observations (Fisher and Walsh, 1974; Spencer et al., 1976a) and Hersh and Jorns have conducted parallel and complementary work on deazaflavins concurrently (Jorns and Hersh, 1975; Hersh and Jorns, 1975).

Experimental Procedure

Materials. Riboflavin, FAD, NAD^+, NADH, $NADP^+$, NADPH, acetylpyridineNAD^+, acetylpyridineNADH, and brewers' bottom yeast were obtained from Sigma. FMN was from phosphodiesterase cleavage of FAD. Deazariboflavin, deazaFMN, and deazaFAD were obtained in nonradioactive and labeled forms as described in the accompanying paper (Spencer et al., 1976). NADH labeled isotopically with ^2H or ^3H at C-4 was prepared after Oppenheimer et al. (1971); while labeled NADPH was prepared according to Rubenstein and Strominger (1974).

An adaptation of the $CuSO_4$/salicylaldehyde mediated exchange procedure of Ikawa and Snell (1954) was used to prepare [2-^2H]- and [2-^3H]-D,L-alanine, the former greater than 95% ^2H by ^1H nuclear magnetic resonance (NMR) analysis, the latter at 1500 cpm/nmol. [2-^3H]-D,L-Proline was prepared by exhaustive oxidation of D-proline using D-amino acid oxidase to give the cyclic imine, Δ^1-pyrroline-2-carboxylate. Reduction of this species with sodium borotri-

From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received September 24, 1975.* This work was supported in part by National Institutes of Health Grants GM 20011 and GM 21643, by the donors of the Petroleum Research Fund, administered by the American Chemical Society, and by a National Science Foundation predoctoral fellowship (R.S.).

[1] Abbreviations used are: 5-deazaFMN, 5-deazariboflavin 5′-phosphate; 5-deazaFAD, 5-deazariboflavin 5′-diphosphate, 5′ → 5′-adenosine ester.

tiide (163 Ci/mol, New England Nuclear) and chromatography on Dowex 50 gave the radiopure [2-^{3}H]-D,L-proline at 4500 cpm/nmol.

[1-^{3}H]-D-Glucose (4.47 Ci/mmol) was from New England Nuclear and was used after dilution with nonradioactive glucose to 3.31×10^5 cpm/nmol. All other reagents were from commercial sources, used as received at the highest available purity. Instrumentation was as reported (Spencer et al., 1976).

Enzyme Preparations and Assays

Beneckea harveyi NAD(P)H:flavin Oxidoreductase (trivial name, oxidoreductase). Enzyme Assay. Activity is measured in oxygenated 0.10 M sodium pyrophosphate (pH 8.65) buffer at 30 °C, containing 20 μM riboflavin and enzyme to a final volume of 1.00 ml. The reaction is initiated with 200 nmol of NADH and activity monitored at 340 nm. Deazariboflavin reduction is assayed similarly, but at an initial concentration of 100 μM deazariboflavin since deazaflavins turn over stoichiometrically, rather than catalytically. The deazaflavin reduction is monitored at 396 nm, since this long wavelength absorption completely bleaches on reduction ($\Delta\epsilon$ = 12 000) and since dihydrodeazaflavins are inert to autoxidation on the time scale of these assays (Spencer et al., 1976).

Enzyme Purification. Partially purified oxidoreductase was the generous gift of Dr. James Becvar. Cell extracts, containing the oxidoreductase, were isolated and purified by an improved procedure (Baldwin, et al., 1975) over that originally reported (Duane and Hastings, 1975). This newer procedure was devised principally for the purification of the luciferase, and as such the luciferase and oxidoreductase are initially coisolated. This procedure is summarized as follows. Following cell lysis, DEAE-cellulose is added to absorb both enzymatic activities and then is washed with 0.15 M phosphate (pH 7.0) buffer to remove weakly bound protein. The oxidoreductase and luciferase are removed simultaneously by a 0.35 M phosphate (pH 7.0) wash, and the two activities coprecipitated between 40 and 70% ammonium sulfate saturation. The pellet is collected and dialyzed and the two activities are then separated by the gradient elution of a DEAE-Sephadex A-50 column (Gunsalus-Miguel et al., 1972; Baldwin et al., 1975). The oxidoreductase elutes at a much lower ionic strength than the luciferase, and was provided to us by Dr. Becvar at this point in its purification.

The partially purified oxidoreductase, 800 units (micromoles of NADH oxidized per minute by riboflavin under assay conditions) in 300 ml of buffer, was concentrated by precipitation at 70% ammonium sulfate saturation. The precipitate was collected by centrifugation, and resuspended in the minimal volume of 0.05 M sodium phosphate (pH 7.0) buffer containing $5(10^{-4})$ M dithiothreitol. The enzyme solution (30 ml) was clarified and applied in two portions to a 102 × 5.0 cm Sephadex G-100 column equilibrated with this same buffer. The column was developed at a flow rate of 25 ml/h and the fractions with oxidoreductase activity from the two runs were pooled. To these fractions (200 ml) was added 50 ml of 0.50 M potassium phosphate buffer (pH 7.0) and the enzyme absorbed onto a squat DEAE-cellulose column. The oxidoreductase was removed by elution with 0.25 M phosphate (pH 7.0) buffer and the concentrated fractions (70 ml, 320 units) were dialyzed against 0.10 M phosphate and 10^{-3} M dithiothreitol. For determination of the enzyme activity, a small aliquot was removed and further dialyzed against degassed buffer containing no dithiothreitol. The protein concentration of this sample was estimated by a Lowry assay, and the specific activity thus measured as 6.1 μmol min^{-1} mg^{-1}, with an A_{280} of 0.65 per mg of protein per ml.

At this point in its purification, the oxidoreductase is not homogeneous as determined by polyacrylamide electrophoresis, containing one major and two minor protein contaminants.

Enzyme Properties. The enzyme is purified in sodium phosphate buffer (pH 7.0) containing 10^{-3} M dithiothreitol. While this pH is not optimum for enzyme activity, it represents the pH condition of maximum enzyme stability. The maintenance of activity is absolutely dependent on the presence of dithiothreitol (or dithioerythritol) during purification and storage, although during purification this activity loss is reversible. Following purification, the oxidoreductase is completely stable in solution at 4 °C for several weeks. The oxidoreductase may be frozen for brief periods and then thawed without activity loss; however, freezing apparently adversely affects the enzyme's stability, with decreased activity apparent several days after thawing. Prolonged storage is possible in liquid nitrogen (>50% recovery of activity following rapid thawing and dialysis, after 1 year). The oxidoreductase is stable to dilution and to lyophilysis, and retains activity for several hours at 30 °C in the presence of substrates. Addition of exogenous protein (albumin) does not increase the specific activity.

Gerlo and Charlier (1975) have also reported purification of this oxidoreductase by a similar procedure, obtaining the enzyme at a very slightly lower specific activity. We can offer no explanation for the differences in enzyme properties (particularly with regard to the ability of the lower molecular weight reductase to use NADPH) these workers have observed; this may reflect a heterogeneity within the bacterial strain.

Old Yellow Enzyme. Old Yellow Enzyme (yeast NADPH:(acceptor) oxidoreductase, EC 1.6.99.1) was obtained from an autolysate of brewers' bottom yeast by the affinity column method of Abramovitz and Massey (personal communication). The enzyme had an absorbance ratio at 280 and 462 nm of 11.7 and a turnover number (with oxygen) of 26 min^{-1} (μmol of NADPH/μmol of FMN, 0.1 M phosphate (pH 6.65) at 25 °C). Disc gel electrophoresis of material directly from the affinity column exhibited a single protein band, although on standing limited proteolysis appeared to ensue. Thus the enzyme was quick frozen on elution from the affinity column and then thawed as required. Apoenzyme was prepared by dialysis against 2 M KBr, 0.2 M phosphate (pH 5.3), 0.3 mM ethylenediaminetetraacetate, 10 μM phenylmethanesulfonyl fluoride, 4 °C, four times 200 volumes, followed by dialysis against 20 mM pyrophosphate (pH 8.5), 4 °C to remove KBr (V. Massey, personal communication). The apoenzyme was obtained in high yield with typically less than 3% residual holoenzyme activity, and the full specific activity was recovered on reconstitution with excess FMN. Reconstitution with deazaFMN was effected by addition of a slight excess of deazaFMN to apoenzyme followed by dialysis (PP_i buffer above) at 4 °C for 6 h. The reconstituted enzyme was ca. 50% loaded with deazaFMN (by absorbance at 280 and 404 nm) and was assayed in the above pyrophosphate buffer at 12 °C to avoid thermal denaturation.

D-Amino-Acid Oxidase. D-Amino-acid oxidase (D-amino-acid:oxygen oxidoreductase (deaminating), EC

Table I: *B. harveyi* NAD(P)H:(flavin) Oxidoreductase–Substrate Specificity for Flavin Reduction.[a]

Substrate Pair	Rel Initial Rates	Substrate Pair	Rel Initial Rates
NADH–riboflavin	1.00	NADPH–riboflavin	0.80
NADH–FMN	1.00	NADPH–FMN	0.12
NADH–FAD	0.25	NADPH–FAD	0
NADH–deazariboflavin	0.0076	NADPH–deazariboflavin	0.0008
NADH–deazaFMN	0.0053		
NADH–deazaFAD	0		

[a] Riboflavin reduction was assayed as described in the Experimental Procedure, with 0.055 unit of enzyme giving an initial velocity of 0.35 OD min^{-1} at 340 nm. Direct reduction of FMN and FAD was observed anaerobically, eliminating the possibility of turnover resulting from riboflavin contamination. With deazariboflavin, the enzyme concentration was increased tenfold, giving an initial velocity of 0.04 OD min^{-1} at 396 nm.

1.4.3.3) from hog kidney cortex was obtained by the procedure of Brumby and Massey (1968) with a specific activity of 14 μmol per min per mg (NADH consumed with excess lactate dehydrogenase present, 10 mM D-alanine, 2.5 μM FAD, 0.1 M $NaPP_i$, pH 8.5, 30 °C) and gave a single band on disc gel electrophoresis, with an absorbance ratio at 274 and 462 nm of 10.3. Apoenzyme was prepared after Massey and Curti (1966) and was reconstituted with excess deazaFAD and dialyzed 6 h vs. 20 mM potassium pyrophosphate (pH 8.5) and 1 m*M* dithioerythritol at 0 °C (L. Hersh, personal communication). The deazaFAD-enzyme obtained was ca. 70% loaded (by absorbance ratio) and contained 0.005% residual FAD holoenzyme, as assayed by release of tritium to solvent from [2-^{3}H]-D,L-alanine. All experiments were performed in the above reconstitution buffer at 20 °C.

Glucose Oxidase. Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* was obtained from Sigma (type VI) and used without further purification. The enzyme had an absorbance ratio at 278 and 451 nm of 11.0, a specific activity of 15 μmol per min per mg (O_2 consumption in 0.1 M P_i, pH 6.1, 25 °C), and exhibited a single major and one minor band on disc gel electrophoresis. Apoenzyme was prepared by four ammonium sulfate precipitations at pH 1.4 (Swoboda, 1969) followed by one at pH 6.1, for a typical yield of 35% with full specific activity recovered on reconstitution with excess FAD. Reconstitution with excess deazaFAD followed by ammonium sulfate precipitation yielded fully loaded deazaFAD-enzyme that was stable to dialysis or gel filtration, and remained intact for at least a week at 4 °C. All experiments were performed at 20 °C in 0.1 M phosphate (pH 6.1).

Results

Enzymatic Redox Reactions with 5-Deazariboflavin. In general the vitamin B_2, riboflavin, is not the active form of the flavin cofactors, but must be processed enzymatically to the coenzyme forms FMN and FAD. The same expectation would hold for 5-deazariboflavin. However, we have noted in a recent preliminary report (Fisher and Walsh, 1974) that the NAD(P)H:(flavin) oxidoreductase from *Beneckea harveyi* will reduce both riboflavin and 5-deazariboflavin. This organism is a luminescent marine bacterium and the presumed physiological role of the oxidoreductase is as part of the electron transport chain of the light-producing process. The oxidoreductase continuously funnels dihydroisoalloxazine molecules ($FMNH_2$) to the active site of the luciferase, which then uses O_2 and the coupled oxidation of a long chain aldehyde to result in the emission of light from some excited state of the flavin chromophore (Baldwin et al., 1975). The oxidoreductase is unique in utilizing flavins as substrates rather than as coenzymes bound in stoichiometric amounts. Thus, only catalytic quantities of enzyme are required for obtaining a detailed comparison of the behavior of the flavin analogue and the normal isoalloxazine. Furthermore, studies with this enzyme are free of the various difficulties associated with the preparation and reconstitution of apoenzymes (as is necessary for virtually all other flavoenzymes).

Purification of the enzyme as indicated in Experimental Procedures yields an oxidoreductase preparation purified approximately 100-fold over initial extracts. The key step is gel filtration, which due to the small size of the enzyme (molecular weight equal to 24 000; Duane and Hastings, 1975) results in a 20-fold purification during this step alone. However, the enzyme is not homogeneous at this stage. Polyacrylamide gel electrophoresis (5% bisacrylamide) produces a gel where the enzyme activity migrates with the dye marker (bromophenol blue). At 6% bisacrylamide the mobility of the oxidoreductase is retarded, and the oxidoreductase moves as two close running bands, each containing identical NADH (and NADPH) oxidase activities, suggestive of isoenzymes. The purity of the enzyme is approximately 25%, with the contaminating proteins having no detectable effect on the oxidoreductase activity.

Anaerobic assay of the oxidoreductase, monitoring riboflavin reduction at 445 nm, yields a specific activity that is reproducibly 0.85 that of the aerobic specific activity. This slightly higher turnover number in oxygenated buffer suggests that intervention of molecular oxygen at the active site to oxidize dihydroriboflavin can accelerate catalysis marginally. The anaerobic specific activity better resembles the conditions under which deazaflavins are assayed and the comparisons of specific activities presented in Table I are calculated relative to this number.

Deazariboflavin behaves as a competitive inhibitor (K_i = 8 μM) to the oxidoreductase catalyzed reduction of riboflavin by NADH, while at higher enzyme concentrations, and in the absence of riboflavin, deazariboflavin itself undergoes reduction at a V_{max} 0.0076 that of the anaerobic reduction of riboflavin. The K_m observed for deazariboflavin (7 μM) is somewhat larger than that for riboflavin (K_m = 0.82 μM, under catalytic conditions), supporting deazariboflavin as a recognizably potent analogue of riboflavin.

Establishment of Redox Equilibrium and Determination of Deazaflavin Redox Potentials. The reduction of deazariboflavin by NADH in the presence of oxidoreductase proceeds to an equilibrium position (K_{eq} = [deazaRFH$^-$][NAD$^+$]/[deazaRF] [NADH] = 1.7 ± 0.2), as opposed to riboflavin which is quantitatively reduced under anaerobic conditions. DeazaFMN, a slightly poorer substrate for the oxidoreductase (vide infra), exhibits a different equilibrium constant (K_{eq} = 1.0 ± 0.2). That an equilibrium position is indeed reached is confirmed by the ability to manipulate the equilibrium position by the incremental adjustment of the reactant concentrations (Figure 1). The equilibrium is disrupted, as would be predicted, by the addition to the assay of recycling systems designed to selectively remove one of the reactants. Utilization of buffer con-

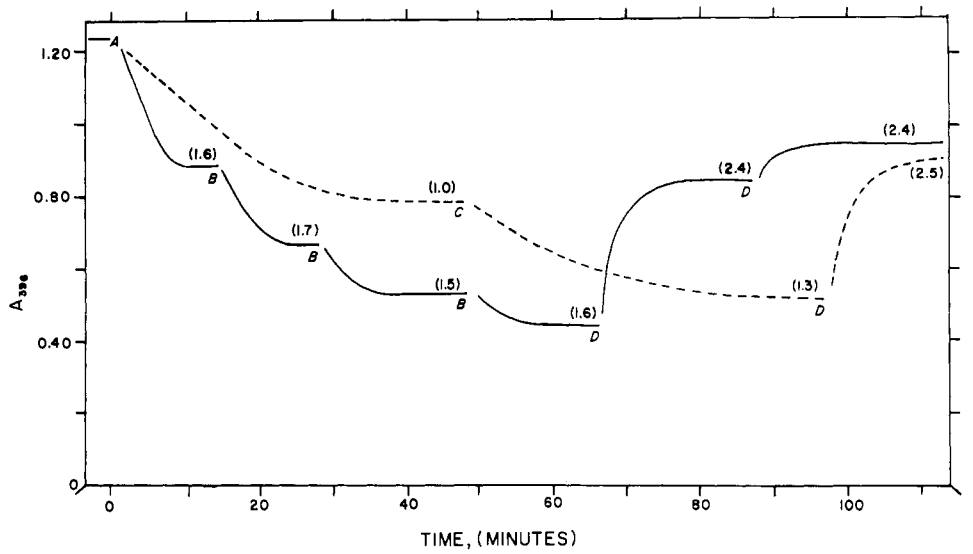

FIGURE 1: An equilibrium study of the *B. harveyi* oxidoreductase catalyzed reaction between NADH, and 4(*R*)-[^{2}H]NADH, with 5-deazariboflavin. Each incubation contained at the start 1.23 A_{396} (103 nmol) of deazariboflavin and 0.72 unit of enzyme (μmol min^{-1}, with NADH and riboflavin) in 1.00 ml total of 0.10 M sodium pyrophosphate buffer (pH 8.65) equilibrated to 30 °C. Point A represents initiation of both reactions with 33 nmol of NADH (—), and 54 nmol of 4(*R*)-[^{2}H]NADH (- - -), respectively. Points B are further incremental additions of NADH; point C is a second addition of the 4(*R*)-[^{2}H]NADH. Points D represent addition of 730. nmol of NAD^+ to each cuvette. The calculated equilibrium constants, corrected for volume changes, are given above each equilibrium position. The difference in equilibrium constants for the NADH reaction and the 4(*R*)-[^{2}H]NADH reaction does not measure an equilibrium isotope effect, since the disproportionation reaction rapidly distributes the deuterium label among the two prochiral dihydrodeazaflavin C-5 positions.

taining 5% ethanol and alcohol dehydrogenase (which at pH 8.65 will regenerate NADH from NAD^+) allows the reaction to proceed slowly to complete deazariboflavin reduction. On the other hand, as a result of the low K_m for riboflavin and the large quantities of enzyme needed to effect rapid deazariboflavin reduction, the presence of trace amounts of riboflavin will rapidly and irreversibly consume the NADH, and thus drive the reaction toward oxidized deazariboflavin. The back reaction between NAD^+ and 1,5-dihydrodeazariboflavin is independent of the presence of oxygen, consistent with the slow autoxidation of the reduced deazaisoalloxazine system (Spencer et al., 1976). As may be seen from Figure 1, the rate of the reverse reaction, with less than saturating NAD^+ and in the presence of NADH and deazaRF, is some sixfold faster than the initial rate of deazariboflavin reduction by NADH. With optimal conditions (no deazaRF initially, a system to remove NADH) the rate of oxidoreductase catalyzed oxidation of deazaRFH$^-$ by NAD^+ is 35 times greater than the V_{max} in the opposite direction. This rate represents a deazaflavin redox reaction proceeding at 27% the rate of the normal enzyme-catalyzed reaction, indicating that the kinetics of enzyme-catalyzed deazaflavin redox reactions may on occasion approach that of flavins. Interestingly, this occurs for a reaction completely opposite in direction (NAD^+ reduction) to that of the enzyme in vivo (flavin reduction).

The fact that the enzyme-catalyzed reaction between the deazaflavins and the dihydropyridine ring of NADH exhibits an equilibrium constant near unity (1.7–3.5 for deazaRF) indicates that the redox potential of the deazaRF-deazaRFH$^-$ couple is essentially identical (-0.310 ± 0.005 V) to that of the NADH-NAD^+ couple ($E_0' = -0.320$ V; Loach, 1968). Given the similarity in structure between the reduced forms of each, this redox potential similarity is perhaps not too surprising. Blankenhorn (1975) has estimated a lower value (−0.380 V) from electrochemical measurements. While the value obtained from this enzyme equilibrium is the better estimate, it is immediately apparent that the deazaflavin redox couple is significantly more negative than the flavin couple (Old Yellow Enzyme, $E_0' = -0.122$ V, riboflavin, $E_0' = -0.208$ V; Loach, 1968). This information has predictive value in determining the preferred direction of substrate-deazaflavin interaction. For example, amino acids and hydroxy acids, which are oxidized by a number of flavoenzymes, as a result of their redox potentials (lactate, $E_0' = -0.185$ V; Loach, 1968) will have an equilibrium with deazaflavins very much favoring instead the formation of hydroxy or amino acid and oxidized deazaflavin. This tendency is in fact observed with several of these deazaflavin-reconstituted flavoenzymes (Averill et al., 1975; Hersh and Jorns, 1975; this manuscript).

The N-1 Anion of Deazariboflavin as the Substrate for Oxidation by NAD^+. The oxidoreductase catalyzed reduction of deazariboflavin with NADH shows a broad pH optimum between pH 8.2 and 9.7, with activity decreasing to ca. 50% between pH 6.0 and 7.0. The reverse reaction, however, shows a similar optimum (pH 8.6–9.1) but a rapid decrease with decreasing pH to ca. 5% at pH 6.0. These data suggest that it is the dihydrodeazariboflavin N-1 anion ($pK_a = 7.2$) that is oxidized, as is the case for nonenzymatic oxidation of dihydrodeazaflavins by riboflavin and oxygen (Spencer et al., 1976).

Substrate Specificity and Rate-Determining Step in Catalysis. We have also determined the substrate specificity of this enzyme (Table I). The oxidoreductase tolerates a wide variety of substrate pairs with varying degrees of efficiency. It is apparent from inspection of these data that the steric demands on the active site by isoalloxazines and deazaisoalloxazines are quite different. In general, the oxidoreductase is less accommodating to deazaflavins. This is particularly clear with deazaFAD, which is not an enzyme substrate, while FAD is (with NADH). Indeed, part of the explanation for the lower turnover numbers for deazaflavins may be that the active site does not maximize the proper donor-

acceptor orbital overlap required for electron transfer in the productive deazaflavin–dihydronicotinamide intermolecular complex.

As a further mechanistic probe of the reaction, we have examined the kinetic isotope effects for the direct hydrogen transfer from NADH to the flavins. Preparation of 4(R)-[^{3}H]NADH and incubation with oxidoreductase and riboflavin indicated stereospecific transfer of this hydrogen, as indicated by the complete volatilization (>90%) of the radioactivity, after prolonged incubation. This stereospecificity was confirmed by the kinetics observed with 4(R)-[^{2}H]NADH, which exhibited an unambiguous primary isotope effect equal to 4.8 ± 0.1, measured both aerobically at 340 nm (NADH oxidation) and anaerobically at 445 nm (riboflavin reduction). A small, possibly secondary isotope effect of 1.2 ± 0.1 with 4(S)-[^{2}H]NADH was seen for riboflavin reduction.

Use of deazariboflavin had no effect on the NADH stereospecificity; a kinetic isotope effect of 3.3 ± 0.1 was observed only for the 4(R)-[^{2}H]NADH, while deazariboflavin reduction with the 4(S) isomer gave a rate identical with the protio control. DeazaFMN has identical kinetic isotope effects. The reverse reaction, reduction of NAD^+ by deazaRFH$^-$, also has a deuterium isotope effect. With [5-$^2H_1$]deazaRFH$^-$, which is necessarily achiral as a result of the rapid deazaflavin disproportionation (Spencer et al., 1976), the observed effect is 1.6 ± 0.2. Utilization of [5-$^2H_2$]deazaRFH$^-$ (which obligates the transfer of a deuterium, regardless of disproportionation) increases the kinetic isotope effect to 2.8 ± 0.2. Thus, even though a large difference in absolute rate exists between riboflavin and deazariboflavin, hydrogen transfer remains rate determining for both. These data eliminate the possibility of substrate binding, or product release from the enzyme as the slow steps of catalysis in either case.

Examination of Stereochemistry of Enzyme-Catalyzed Hydrogen Transfer. Dihydrodeazaflavins with tritium at C-5 are potentially chiral at this locus; retention or transfer of tritium in the oxidoreductase-catalyzed NAD^+ oxidation of these species should provide an assay of this chirality. Chirality of the oxidoreductase with respect to nicotinamide was established as 4(R) in the forward direction (vide supra) and in the reverse direction, since 5(R,S)-[^{3}H]deazaRFH$^-$ (from NaB^3H_4 reduction), mixed with NAD^+ and oxidoreductase, yielded [4-^{3}H]NADH exclusively labeled in the 4(R) position since tritium was rendered volatile with alcohol dehydrogenase and acetaldehyde, but remained nonvolatile when lipoamide dehydrogenase was the assaying enzyme.

[5-^{3}H]Dihydrodeazaflavin samples were prepared with the oxidoreductase, D-amino-acid oxidase, and glucose oxidase, using both deazaflavin and tritiated substrate, and [5-^{3}H]deazaflavin and nonradioactive substrate, to yield, presumably, samples of both C-5 epimers. In assay with oxidoreductase and NAD^+, however, all [5-^{3}H]dihydrodeazaflavins showed partial transfer of label into NADH and preferential retention in deazaflavin. This led to the conclusion that the lack of dihydrodeazaflavin chirality was independent of enzymatic control. Subsequent nonenzymatic experiments with [2-^{14}C]deazariboflavin led to the discovery of the facile deazaflavin disproportionation reaction noted in the accompanying paper (Spencer et al., 1976).

Enzymatic Experiments with 5-DeazaFMN. In addition to its ability to function as a freely dissociable substrate for the *B. harveyi* oxidoreductase, deazaFMN has recently been used in stoichiometric amounts with the apoproteins of the *Azotobacter* flavodoxin (Edmonson et al., 1972), *N*-methylglutamate synthetase (Jorns and Hersh, 1975), and L-lactate oxidase (Averill et al., 1975). In the flavodoxin reconstitution no assay of catalytic competence could be determined. In the synthetase deazaFMN functions catalytically; in the L-lactate oxidase it undergoes stoichiometric reduction but not O_2 reoxidation. In both cases α-^{3}H substrates produce tritiated dihydrodeazaFMNH$^-$ on enzymatic reduction.

We now report reconstitution of yeast Old Yellow Enzyme with deazaFMN and its reduction with NADPH. We chose to examine this enzyme as a representative flavoprotein dehydrogenase whose purification and reversible resolution have been documented. However, we note at the outset that reconstitution of apo Old Yellow Enzyme with the deazaisoalloxazine displayed many of the technical problems noted recently with lactate oxidase reconstitution (Averill et al., 1975): poor and variable yields of deazaFMN-reconstituted enzyme. The Old Yellow Enzyme situation is further exacerbated by a low affinity of apoenzyme for deazaFMN. FMN reconstitutes activity of our apoenzyme with a K_m of 0.30 μM and a turnover number of ca. 30 min^{-1} (with O_2, 25 °C).

When apo Old Yellow Enzyme is added to solutions of deazaFMN, a K_D of 3–6 μM can be estimated by quenching of deazaflavin fluorescence at 459 nm[2] (Spencer et al., 1976). The absorption spectrum of deazaFMN bound to Old Yellow Enzyme shows a red shift of 8 nm, from 396 to 404 nm, similar to that noted for *N*-methylglutamate synthetase (Jorns and Hersh, 1975) and lactate oxidase (Averill et al., 1975). On addition of saturating amounts of NADPH to the deazaFMN-enzyme solution, there is no incremental increase in NADPH oxidation over the rate due to contaminating FMN holoenzyme in the apoenzyme preparation (typically 1–3%). The lack of reactivity of deazaFMNH$^-$-enzyme with O_2 is now expected (Spencer et al., 1976) but even when ferricyanide was the electron acceptor, turnover with the deazaFMN-enzyme was not detected, so Old Yellow Enzyme reconstituted with deazaFMN also does not function catalytically as a dehydrogenase with this one-electron acceptor.

On the other hand, NADPH does stoichiometrically reduce deazaFMN bound to Old Yellow Enzyme, with the rate of reduction being slow and the extent variable. We have seen rates of reduction varying from 0.03 to 0.8 min^{-1} and reduction proceeding anywhere from 25 to 100% completion. Presently, the causes for this frustrating variability are unclear. Even the lowest turnover numbers are clearly due solely to the enzymatic reduction since no detectable nonenzymatic reduction of deazaFMN by NADPH occurs during the course of these experiments. The rate of reduction of deazaFMN-enzyme is from 50- to 1000-fold slower than native enzyme. Reoxidation of dihydrodeazaFMN-Old Yellow Enzyme, prepared from NADPH, has not been achieved despite addition of a large excess of $NADP^+$; this may be an equilibrium effect. Borohydride-reduced deazaFMNH$_2$-enzyme is partially reoxidized by excess $NADP^+$ (30% oxidation). We have not pursued the Old Yellow Enzyme experiments further due to these technical variabilities.

Enzymatic Experiments with 5-DeazaFAD: In the ac-

[2] Professor V. Massey has found this K_d to be approximately 4 μM by the same technique.

companying paper we describe the preparation of 5-deaza-FAD and now have examined the interaction of this coenzyme analogue with three FAD-requiring enzymes, D-amino-acid oxidase from hog kidney (EC 1.4.3.3), glucose oxidase from *Aspergillus niger* (EC 1.1.3.4), and *E. coli* glyoxalate carbo-ligase (EC 4.1.1.47). We discuss experiments with the two oxidases below. Reconstitution of glyoxalate carboligase with deazaFAD, which functions in a nonoxidative role in this instance, is reported elsewhere (Cromartie and Walsh, 1976).

(A) D-Amino-Acid Oxidase. We have analyzed this oxidase because it is among the best characterized flavoenzymes mechanistically. In addition to normal oxidative deamination of D-α-amino acids, the enzyme carries out α,β elimination of HCl from β-chloro-α-amino acid substrates (Walsh et al., 1971, 1973) and the oxidation of nitroalkane anions (Porter et al., 1973) alluded to in the accompanying paper. Experiments similar to those reported here have been done independently and concurrently by Hersh and Jorns (1975, 1976).

Reconstitution of apo D-amino-acid oxidase with deaza-FAD is effected either by dialysis or ammonium sulfate precipitation to remove excess deazaFAD. One can monitor perturbation of the bound deazaflavin spectrum on addition of benzoate or anthranilate, compounds shown by Massey and Ganther (1965) to have marked effects on the native holoenzyme visible spectrum. Addition of 0.15 m*M* benzoate to deazaFAD-enzyme results in a perturbation of deazaflavin spectrum (λ_{max} shifted from 403 to 407 nm, shouldering from 425 to 435 nm) which precisely parallels, though blue shifted, the spectroscopic changes seen with FAD-enzyme. The essential identity of vibronic changes suggests very similar interactions between benzoate and either the isoalloxazine or deazaisoalloxazine in the enzyme active site. Addition of 0.15 mM sodium anthranilate causes slight but real spectral changes, most notably a long wavelength shoulder on the 403-nm peak; this in contrast to the native FAD-enzyme with anthranilate where a strong charge transfer interaction occurs. The absence of such pronounced long wavelength absorption in the deazaFAD-enzyme complex most likely implicates the N-5 lone pair electrons in the normal coenzyme (and its E_0') as important in that charge transfer species.

Catalytic competence of deazaFAD reconstituted D-amino-acid oxidase was investigated, first with D-alanine as substrate. Monitoring reduction of deazaflavin at 402 nm, half-times on the order of 50 min are consistently observed, with reduction proceeding to only 25% of total deazaflavin-enzyme (saturating D-alanine, conditions as in Experimental Procedures). This compares with half-times of 18 min found by Hersh and Jorns (1975, 1976), who see almost complete reduction of bound deazaFAD. No oxygen oxidation of reduced enzyme-bound deazaFAD is seen over a period of hours. Addition of pyruvate and ammonium ion, but not pyruvate alone, to partially reduced bound deazaFAD gives rapid ($t_{1/2} < 1$ min) return of oxidized deazaFAD, reflecting the preferred equilibrium (Hersh and Jorns, 1975), as expected from the low redox potential of deazaflavins. Similar rapid oxidation of bound deazaFADH_2 is seen with ammonium ion and phenylpyruvate or chloropyruvate. Hersh and Jorns (1975) have also seen oxidation with α-iminobutyrate. Use of [α-^{2}H]-D,L-alanine vs. [α-^{1}H]-D,L-alanine (each 13 mM) gives a deuterium kinetic isotope effect of 1.3, comparable to the 1.67 value of Hersh and Jorns (1975, 1976).

In light of the strong evidence for the existence of substate carbanions in catalysis by D-amino-acid oxidase (Walsh et al., 1973; Porter et al., 1973), we sought evidence of reversible formation of carbanion by washout of tritium from [2-^{3}H]-D,L-alanine into solvent, catalyzed by deaza-FAD–D-amino-acid oxidase. Unfortunately, the level of residual FAD holoenzyme, estimated at 1 molecule per 20 000 of deazaFAD enzyme, remains 100-fold too high to probe this point. A rate of 0.45 nequiv of ^3H_2O released min^{-1} (coincident with 0.010 nmol of deazaFAD–enzyme reduced min^{-1}, both linear over 70 min) is entirely accounted for in the control with apoenzyme and no deazaFAD.

The slow rate with D-alanine as substrate prompted us to use D-proline (20-fold higher V_{max} with native enzyme; Massey and Gibson, 1964) as reductant. Indeed, at 1.6 mM D,L-proline, the $t_{1/2}$ for pseudo-first-order reduction is 0.63 min, compared to 3.5 min at 20 mM D,L-proline seen by Hersh and Jorns (1975). Both we (29% reduction) and they (33% reduction) see incomplete reduction of bound deaza-FAD with this amino acid substrate. We note that even at our fast rate, the reduction rate of deazaFAD is some 10^5 slower than reduction of FAD bound in native holoenzyme (Massey and Gibson, 1964).

In view of recent experiments indicating that an active site base of D-amino-acid oxidase can sequester the substrate α hydrogen during catalysis (Walsh et al., 1971, 1973), we wished to determine whether the α hydrogen of proline would end up in H_2O or at carbon 5 of the coenzyme analogue when the deazaFAD–D-amino-acid oxidase was reduced. Incubation of 26 nmol of deazaFAD–enzyme with 1.6 mM [α-^{3}H]-D,L-proline (4500 cpm/nmol) produced 7.5 nmol of deazaFADH_2–enzyme, which, after Sephadex G-25 gel filtration, contained a peak of radioactivity (4600 cpm) coeluting with the enzyme. If all the deaza-FADH_2 enzyme molecules were reduced by direct ^{1}H or ^{3}H transfer, this implies a product isotope discrimination of 6.7, in contrast to the lack of isotope effect seen with native holoenzyme (data not shown). It is mechanistically germane to inquire whether any substrate-derived ^{1}H or ^{3}H ends up in solvent, but the determination of this important point is obscured, as noted above, by the fact that even the one native holoenzyme molecule remaining among 20 000 deazaFAD–enzyme molecules is sufficient impurity (it functions catalytically and at 10^5 the rate of deazaFAD-enzyme) to release excess tritium into solvent. However, Hersh and Jorns (1975) found the k_H/k_D of 1.67 with [α-^{2}H]alanine and a product discrimination of 2.0 with [α-^{3}H]alanine by isolation of the [^{3}H]deazaFADH_2-enzyme. This mirrors the k_H/k_T predicted from the size of $k_H.k_D$ and suggests that the direct hydrogen transfer to deazaFAD is indeed complete. A product tritium discrimination greater than 2.0 would be consistent with some loss of tritium to solvent.

(B) Glucose Oxidase. A second FAD-utilizing enzyme satisfying the criteria of ready availability and of reversible apoenzyme formation and reconstitution is glucose oxidase from *Aspergillus niger*. In particular we hoped that catalysis with deazaFAD in this instance would be sufficiently rapid and complete[3] in our hands to perform some experiments on the relative stereochemistry of hydrogen transfer with the coenzyme analogue.

[3] As a result of the E_0' for the glucose–gluconolactone couple (−0.364 V; Loach, 1968).

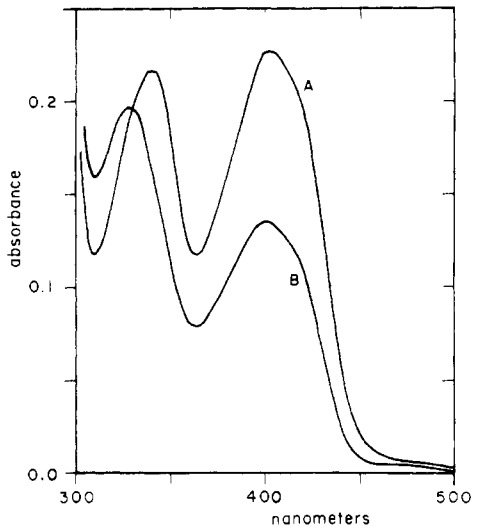

FIGURE 2: The absorption spectra of deazaFAD bound to glucose oxidase. (A) DeazaFAD-glucose oxidase, 5.2 nmol in 300 μl of 0.1 M NaP_i (pH 6.1); (B) stable spectrum achieved within 10 s after the addition of approximately 10 μmol of solid D-glucose.

Apoglucose oxidase can be reconstituted by addition of excess deazaFAD and subsequent ammonium sulfate precipitation to effect complete loading of apoenzyme as judged by the ratio of absorbances at 277 and 402 nm of 8.5. As with reconstituted D-amino acid oxidase there is no obvious vibronic perturbation in the spectrum of deazaFAD when it is bound to glucose oxidase (Figure 2A). Sealed tube denaturation (Jorns and Hersh, 1975) of deazaFAD-enzyme gives an extinction coefficient for the bound deazaflavin of 13 100 M^{-1} cm^{-1} at 402 nm. That the deazaFAD is tightly bound by the apoenzyme is demonstrated by its retention through ammonium sulfate precipitation, extended dialysis, and gel filtration, and that is is displaced only slowly by excess exogenous FAD. Estimation of the association and dissociation rate constants gives an upper limit on the K_d of approximately 0.5 μM.

Addition of solid glucose to deazaFAD-enzyme causes rapid bleaching of the 402-nm absorbance, yielding the spectrum shown in Figure 2B. The reduction achieved is over within 5 s and typically the end point indicates 40–50% of the bound deazaFAD reduced in various preparations. Subsequent addition of more glucose does not generate further reduction, suggesting the position reached is not an equilibrium one. Addition of hydroxylamine (2.5 mM) to partially reduce deazaFAD enzyme in the presence of excess glucose does not produce any spectral perturbation, though with native enzyme this elicits a flavin semiquinone spectrum (Swoboda and Massey, 1966).

Not unexpectedly, no molecular oxygen is consumed when glucose is used to reduce deazaFAD enzyme; the reaction is stoichiometric, not catalytic. $DeazaFADH_2$-enzyme is similarly inert to oxidation by dichlorophenolindophenol (an alternate, though poor, acceptor for native enzyme) or riboflavin. Addition of gluconolactone (in tenfold excess over glucose) produces no detectable reoxidation of $deazaFADH_2$ enzyme, in contrast to the results seen with product reoxidation of other deazaflavin-reconstituted enzymes (vide supra). $DeazaFADH_2$ prepared by borohydride reduction and added to excess apoenzyme yields a fully reduced, tightly bound species, as shown by its lack of absorption at 402 nm and its inertness to oxidation by riboflavin (Spencer et al., 1976). This $deazaFADH_2$-enzyme is rapidly, though only partially (4–7%), reoxidized by gluconolactone (58 mM). Moreover, this small level of oxidized deazaFAD-enzyme is rapidly and completely rereduced on the addition of glucose (14 mM).

The rapid reduction of deazaFAD glucose oxidase by substrate combined with the high K_m (33 mM for glucose with native holoenzyme) have made it difficult for us to estimate the K_m and V_{max} for the reaction with the analogue. For instance, if the K_m for glucose is unchanged in the deazaFAD enzyme, we estimate a half-time for reduction of 0.35 s with saturating glucose. Stopped-flow kinetic experiments will be necessary to resolve these points. Similarly, fast reaction techniques will be required to determine whether the rate-determining step in deazaFAD-enzyme reduction is the hydrogen transfer. This is germane for analyzing the similarity with FAD enzyme since Bright and Gibson (1967) have found that substitution of [1-^{2}H]glucose for glucose as substrate changes the rate-determining step such that FAD reduction now becomes rate determining in overall catalysis.

One can approach the question of direct hydrogen transfer and isotopic discrimination with [1-^{3}H]glucose. Sephadex G-25 gel chromatography of the reaction mixture of [1-^{3}H]glucose and deazaFAD-glucose oxidase yields a peak of radioactivity coincident with the protein. Assuming complete hydrogen transfer and retention of bound $deazaFADH_2$ (which may be inferred from the low K_d for deazaFAD and the absence of any "tailing" of radioactivity in the column elution), the 8.3 nmol of $deazaFADH_2$ produced and 401 000 cpm of tritium eluting with the protein imply a product selection of 6.5 against tritium. The complementary assay for the directness of hydrogen transfer by appearance of tritium in solvent during deazaFAD-glucose oxidase reduction is precluded by the residual 7% FAD-enzyme carried through the apoenzyme preparation. Repetition of the Sephadex experiment with [U-^{14}C]glucose resulted in no detectable label eluting in the protein peak, demonstrating the absence of a stable covalent adduct in catalysis.

Discussion

The ability of 5-deazaisoalloxazine to substitute for the normal isoalloxazine has now been examined with several flavoenzymes. The consistent finding that the bound deazaflavin is capable of enzyme-catalyzed reduction and oxidation by the specific enzyme substrate establishes the deazaisoalloxazine system as a unique mechanistic probe of flavin redox catalysis. There are several insights that our present findings have provided that may be presented with only brief comment. It is apparent that the presence of a nitrogen atom at position 5 is not critical for the stabilization of coenzyme binding, as the deazaflavins are bound by every apoflavoenzyme with which they have been tested. The bound deazaflavin responds to the active site environment in a similar or identical fashion as the flavin, evidenced by similar extinction coefficient changes, bathochromic shifts, and vibronic resolution of the long wavelength absorbance.

The N-5 also is clearly not a requisite feature for coenzyme reduction and oxidation by the four substrate classes thus far examined: dihydronicotinamides, amino acids, hydroxy acids, and hemiacetals (glucose); but nitrogen at position 5 is necessary for rapid oxidation of reduced flavin by oxygen. A complete listing of those enzymes where catalysis of deazaflavin redox reactions have been observed is given

Table II: Properties of Deazaflavin Reconstituted Apoflavoenzymes.[a]

Enzyme	5-Deaza Analogue	Catalyzed Substrate Oxidation	Catalyzed Oxidation of Dihydrodeazaflavin by O_2	Catalyzed Oxidation of Dihydrodeazaflavin by Product	Transfer of Substrate ^3H to Deazaflavin	Ref
Beneckea harveyi NAD(P)H:(flavin) oxidoreductase	DeazaRF, deazaFMN	+	−	+	+	This manuscript
Yeast NADPH:(acceptor) oxidoreductase (old yellow enzyme)	DeazaFMN	+	−	*b*	+	This manuscript Massey, personal communication
Mycobacterium smegmatis L-lactate monooxygenase (decarboxylating)	DeazaFMN	+	−	+	+	Averill et al. (1975)
Pseudomonas MA *N*-methyl glutamate synthetase	DeazaFMN	+	−	+	+	Jorns and Hersh (1975)
Hog kidney D-amino acid:O_2 oxidoreductase (deaminating)	DeazaFAD	+	−	+	+	Hersh and Jorns, (1975); this manuscript
Aspergillus niger β-D-glucose:O_2 oxidoreductase	DeazaFAD	+	−	*b*	+	This manuscript
Yeast succinate:(acceptor) oxidoreductase	DeazaFAD	+	*c*	*c*	*c*	Grossman et al. (1975)
Milk xanthine:O_2 oxidoreductase	DeazaFAD	+	−	*c*	*c*	Massey, personal communication

[a] A (+) indicates a positive observation, although the extent and rate is a function of the individual enzyme. A (−) indicates no enzymatic catalysis. Only those enzymes where an enzyme-catalyzed deazaflavin redox reaction has been observed are included in the above table. Deazaflavins have been found to bind to the following additional apoflavoenzymes: deazaRF, to egg white riboflavin binding protein (J. Becvar and V. Massey, personal communication); deazaFMN, to *Azotobacter vinelandii* flavodoxin (Edmondson et al., 1972; Edmondson and Singer, 1975), to *Peptostreptococcus elsdenii* flavodoxin (Burnett et al., 1974), to rabbit liver pyridoxamine 5′-phosphate:O_2 oxidoreductase (deaminating) (Kazarinoff and McCormick, 1975), and to microsomal NADPH:cytochrome *c* oxidoreductase (Edmondson and Singer, 1975); deazaFAD to *Escherichia coli* glyoxylate carboligase (dimerizing and reducing) (Cromartie and Walsh, 1976). [b] Only partial oxidation of nonenzymatically reduced deazaflavin. [c] Not yet determined.

in Table II. From the information accumulated it does not appear likely that, as was originally hoped, a stable substrate–deazaflavin intermediate will be detected with any enzyme. Deazaflavins have allowed the first unequivocal demonstration of direct, and reversible hydrogen transfer from substrate to flavin. While this is important mechanistically, we are limited in the extent to which we may interpret our enzymatic data by the paucity of information concerning the chemistry of the deazaflavin system. Nevertheless, we feel justified in utilizing these data as a basis for extending our knowledge of flavoenzyme catalysis.

In this context, some direct discussion is in order with respect to the contention recently voiced by Hemmerich and Jorns (1973) and Blankenhorn (1975), to the effect that the structural similarities between dihydrodeazaflavins and dihydronicotinamides render deazaflavins as models only for dihydronicotinamide, and not flavin, redox reactions. In light of the information presented in these manuscripts, a more accurate statement may be that deazaflavins are a model for both. Certainly some fundamental similarities exist for dihydronicotinamides and dihydrodeazaflavins: hydrogen transfer occurs to and from the C-4 position of a dihydropyridine, without exchange with solvent (Brüstlein and Bruice, 1972; Shinkai and Bruice, 1973; Spencer et al., 1976); both have similar redox potentials (although a flavin redox potential is very much a function of the protein active site; note the very low potentials of flavodoxins); both have unstable one electron oxidized forms and are only very slowly oxidized by oxygen. However, beyond these parallels are differences that require consideration of dihydrodeazaflavins as separate chemical systems apart from, but related to, dihydronicotinamides. Dihydrodeazaflavins are oxidized only as their N-1 anions (ionization adjacent to the dihydropyridine ring is not possible for dihydronicotinamides), disproportionate far more readily, and are oxidized significantly faster by several electron acceptors (e.g., ferricyanide and riboflavin). While the similarities might appear to outweigh the differences, the validity of deazaflavins as legitimate mechanistic probes of flavin catalysis is firmly established by the enzyme chemistry. The ability of the active site to catalyze electron transfer for both flavins and deazaflavins carries an implication that cannot be overemphasized: that deazaflavins are able to utilize to their own advantage the identical features at the active site that exist only for the purpose of accelerating electron transfer to the normal flavin coenzyme. This is strong circumstantial evidence for an identical mechanism operating, perhaps not only between deazaflavins and flavins but as well between flavins and dihydronicotinamides. This point is discussed directly.

We have investigated two reaction classes with deazaflavins: those using dihydronicotinamide substrates and those involving carbonyl-related compounds. As distinct mechanisms have been proposed for these categories, the mechanistic implications for each of the deazaflavin reactivity will be discussed separately.

The nature of electron transfer from dihydronicotinamides has long been enigmatic. Within recent years, numerous model system studies have been interpreted as indicating hydride transfer to electron deficient aromatics (Kurz and Frieden, 1975), carbonyls (Dunn and Hutchison, 1973; Shore et al., 1974; Sund, 1968), and isoalloxazines (Wu et al., 1970). Despite hydride transfer having become the usual postulate, it retains an enormous lack of appeal in having no proven precedent among the known chemistry of alcohols and dihydropyridines. For these reasons Hamilton (1971) was led to suggest a more plausible mechanism for the dihydronicotinamide reduction of isoalloxazines involv-

ing intramolecular transfer of two electrons and a proton, concerted with decomposition of a covalent adduct. However, the intermediacy of such a species has yet to be detected, and such a mechanism would appear to be inconsistent with the repeated observation of pH independence and direct hydrogen transfer in nonenzymatic dihydronicotinamide reductions. While these experimental observations would appear to eliminate hydrogen transfer as a proton, and favor hydride transfer, there remains a viable alternative to both. The concept of dihydronicotinamide reactions proceeding by separate electron and hydrogen atom transfer has on numerous occasions been mentioned (e.g., Bruice and Benkovic, 1966; Klinman, 1972; Kosower et al., 1973; Williams et al., 1975), but has rarely received serious consideration. As Klinman has pointed out, many of the arguments for hydride transfer are equally applicable to hydrogen atom transfer. It should be kept in mind that several substituted pyridiniums are one-electron reduced to yield very stable neutral radicals (Kosower and Poziomek, 1963; Badock et al., 1974), and although nicotinamides are not (Brühlmann and Hayon, 1974) the energetics for formation of an intermediate pyridinyl radical may be quite attainable. This would be even more valid if the intermediate were of the nature of a radical pair with the other electron acceptor. While complete arguments for this hypothesis are beyond the scope of this manuscript, we suggest this as the most feasible route for dihydronicotinamide–deazaflavin (and dihydronicotinamide–flavin) reduction. Given the likelihood of hydrogen atom transfer, and if one requires the intermediacy of the neutral pyridinyl radical and the dihydrodeazaflavin anion as product, the sequence for electron transfer and hydrogen atom transfer becomes well defined. For the reduction of deazariboflavin by NADH, hydrogen atom transfer must precede electron transfer; for the oxidation of dihydrodeazariboflavin by NAD^+ the opposite sequence must occur.

If the one-electron transfer were rapid, this mechanism could well account for the partially rate limiting, direct hydrogen transfer seen in both directions for the enzyme-catalyzed reactions of NADH and deazaflavins. This mechanism also offers the important advantage of being equally applicable to flavins as well as deazaflavins, since it confers on the enzyme active site only the responsibility of creating an environment that would stabilize an NADH–flavin molecular complex.

The reaction of flavins with carbonyl-related compounds may well proceed by a similar mechanism. An impressive amount of evidence has accumulated requiring the formation of α-carbanions in the isoalloxazine dependent dehydrogenation of hydroxy acids and amino acids, for both nonenzymatic (Brown and Hamilton, 1970; Main et al., 1972; Shinkai et al., 1974) and enzymatic pathways (Walsh et al., 1973; Porter et al., 1973). By analogy with the synthetic utility of carbanions in covalent bond formation, Hemmerich and coworkers (Ghisla et al., 1973) have presented a variant of the now less likely mechanism first proposed by Brown and Hamilton (1970). This mechanism utilizes a carbanion-derived covalent substrate–isoalloxazine adduct that heterolytically decomposes to yield carbonyl and dihydroflavin. This mechanism still stands as a chemically attractive explanation of the enzymatic data. The ability of isoalloxazines to oxidize nitroalkane anions (Porter et al., 1973; Yokoe and Bruice, 1975) and sulfhydryls (Yokoe and Bruice, 1975; Loechler and Hollocher, 1975) by intermediate covalent adducts, and the ability of several suicide substrates to irreversibly inhibit flavoenzymes by covalent modification of the coenzyme (Walsh et al., 1971; Porter et al., 1973; Rando, 1973; Chuang et al., 1974; Cromartie and Walsh, 1975) serve as powerful reminders that the active site isoalloxazine is indeed capable of covalent bond formation. Hemmerich et al. (1967), Brüstlein et al. (1971), and Clerin and Bruice (1974) have demonstrated that photochemically formed adducts (of the type postulated for enzyme catalysis) can decompose to reduced flavins. These data require that one be exceedingly cautious in excluding such adducts as mechanistic possibilities in the redox catalysis.

However, our failure to detect covalent substrate–deazaflavin intermediates may have bearing on this question. While the stability of such adducts would be expected to be greater for deazaflavins than flavins,[4] none are observed. If they are short-lived intermediates on the reaction path, then to account for the experimental observation of α-proton removal and ultimate transfer to the C-5 of the deazaflavin one must postulate that the heterolytic decomposition of the adduct occurs to place carbanionic character at C-5 (to allow return of the hydrogen as a proton). It is known that simple dihydropyridines (Fowler, 1972) and dihydroquinolines (Coates and Johnson, 1971) have some intrinsic acidity at the methylene carbon; it remains to be demonstrated that this is sufficient to support a covalent adduct hypothesis. A recent communication by Bruice and coworkers (Williams et al., 1975) has summarized several persuasive arguments against a group transfer mechanism, and for radical pair intermediates in isoalloxazine–carbonyl redox reactions. Unfortunately, there are some similar difficulties in adapting this mechanism to deazaflavins. If one accepts carbanion formation as the first step in deazaflavin reduction, then the succeeding step must be one-electron transfer to form the diradical pair. From the diradical pair, reduction may only be completed by synchronous one electron and proton (*not* a hydrogen atom) transfer to C-5. Again, the feasibility of such a process is yet to be demonstrated. Alternatively, carbanion formation may be a nonproductive process for deazaflavin reduction, with the reaction progressing by sequential hydrogen atom and electron transfer (analogous to dihydropyridines). Nonproductive carbanion formation, unfavorable thermodynamics, and the disadvantages of a carbon relative to a nitrogen participation in radical transfers (Williams et al., 1975) may combine to give a plausible explanation for the slowness of deazaflavin reduction by hydroxy and amino acids.

We had hoped to probe whether deazaflavin-reconstituted enzymes catalyze the α,β elimination of HCl from β-halo substrates seen with both D-amino-acid oxidase (Walsh et al., 1971) and L-lactate oxidase (Walsh et al., 1973). However, neither enzyme carries out elimination or oxidation with β-chloro-D-alanine or β-chloro-L-lactate,[5] respectively, rendering this test for substrate carbanion formation moot. The inertness is related at least in part to the low redox potential of the deaza analogues since deaza$FMNH_2$–lactate oxidase is reoxidized by chloropyruvate[5] as is deaza$FADH_2$–D-amino acid oxidase when ammonia is also present.

Although to date deazaflavins have been tested with sev-

[4] However, we note that while nitroalkane anions add nonenzymatically to deazaflavins, the resulting adducts rapidly decompose (Spencer et al., 1976).

[5] C. Walsh and V. Massey, unpublished results.

eral enzymes (Table II), the versatility of flavins in biochemistry still leaves several areas of flavin catalysis to be investigated. One would predict that deazaflavin reconstitution of an external monooxygenase would result in the deazaflavin being susceptible to catalytic reduction by NAD(P)H, but not to activation of molecular oxygen. In order to achieve a better understanding of the reactivity and stability of deazaflavin semiquinones, it would be of interest to examine the behavior of dihydrodeazaflavins with obligate one-electron transferring flavoenzymes (e.g., flavodoxins and adrenodoxin reductase). Recent evidence supporting the intermediacy of covalent adducts in the nonenzymatic isoalloxazine oxidation of sulfhydryls (Yokoe and Bruice, 1975; Loechler and Hollocher, 1975) provides justification for looking for a similar deazaflavin adduct with such enzymes as glutathione reductase or dihydrolipoamide dehydrogenase. Catalytic turnover of a deazaflavin reconstituted dehydrogenase has yet to be achieved, and this will be necessary in order to look for covalent modification of the deazaflavin by a suicide substrate. The possible utilization of deazaflavins as probes of flavin stereochemistry still remains, provided that experimental conditions be found which suppress deazaflavin disproportionation (such as a pH dependency). Finally, we should point out that deazaflavins have uses beyond the investigation of flavin redox mechanisms. We have recently confirmed a nonredox role for the flavin of a flavoenzyme (glyoxalate carboligase) by observing stimulation of enzyme activity on reconstitution of the apoenzyme with deazaFAD and deazaFADH$_2$ (Cromartie and Walsh, 1976). The extraordinary similarity of deazaflavins and flavins with respect to enzyme binding, along with their markedly different reactivities, suggests possible pharmacological applications of deazaflavins as riboflavin antagonists.

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Electrofocusing and Kinetic Studies of Adult and Embryonic Chicken Pyruvate Kinases†

Kenneth H. Ibsen,* Lowell Murray,‡ and Steven W. Marles

ABSTRACT: Chicken embryos less than 15 days old contain only the K isozyme of pyruvate kinase, which appears to exist in vivo as an R,T conformational set with pI values of 7.2 and 6.6, respectively. Sets of lower pI and higher pI K-isozyme variants also are obtained. Whole embryos of 15 days or more of development show progressively increasing amounts of higher pI, lower $K_{0.5S}$ enzymatic variants. Tissue distribution and kinetic properties suggest that the highest pI form (pH 8.8–9.0) is an M-isozyme analogue. The intermediate forms are postulated to be hybrids. Adult liver extracts contain only the embryonic K isozyme; no evidence for an L-isozyme analogue was obtained. All major forms of the enzymes are compared with respect to saturation by phosphoenolpyruvate in the absence of effector and in the presence of fructose 1,6-diphosphate, alanine, serine, phenylalanine, tryptophan, and/or Mg-ATP.

Rat tissues contain three basic noninterconvertible pyruvate kinases. They are: the K form[1], regarded by Imamura and Tanaka (1972) as the prototype enzyme since it is the fetal enzyme as well as the major isozyme of many normal adult tissues; the L form, found as the major liver enzyme and as a minor kidney cortex enzyme; and the M form, found in differentiated skeletal muscle, brain, and heart. Hybrids may exist in the erythrocyte (Imamura and Tanaka, 1972; Peterson et al., 1974) and kidney (Cardenas et al., 1975b).

Surveys of organs from many other mammals show all to have similar isozyme patterns (Schulz and Sparmann, 1972; Imamura and Tanaka, 1972; Whittell et al., 1973; Balinsky et al., 1973; Carbonell et al., 1973; Osterman and Fritz, 1973; Imamura et al., 1973; Kozhevnikovn, : 1973; Ibsen et al., 1975a; Cardenas et al., 1975b). Thus among mammals, homology may be assumed.

The mammalian L and M isozymes appear to be homotetramers (Kayne, 1973; Cardenas et al., 1973; Cardenas and Dyson, 1973). Hybridization studies show the bovine K isozyme also is likely to be a homotetramer (Cardenas et al., 1975b). Therefore, unless the one isozyme arises from an-

†From the Department of Biological Chemistry, California College of Medicine, University of California, Irvine, California 92717. Supported by National Institutes of Health Research Grant CA-07883.
*To whom correspondence should be addressed.
‡An NSF CAST High School Fellow, Summer 1974.
[1]The K, L, and M nomenclature for pyruvate kinase isozymes is employed in this study. Various nomenclatures have been compared (Osterman et al., 1973; Yanagi et al., 1974; Schloen et al., 1974; Strandholm et al., 1975; Ibsen et al., 1975a).