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Reconstitution of Flavin Enzymes with 1-Carba-1-deazaflavin Coenzyme Analogues†

Rob Spencer, Jed Fisher,‡ and Christopher Walsh*

ABSTRACT: The riboflavin analogue 1-carba-1-deazariboflavin has been converted to 1-deazaFAD enzymatically and the coenzymatic activity of the analogue determined with three flavin-dependent enzymes. The NAD(P)H:flavin oxidoreductase from the marine bacterium *Beneckea harveyi* uses 1-deazariboflavin in the catalytic oxidation of NADH. The V_{max} for 1-deazariboflavin reduction by NADH is 0.025 that for riboflavin reduction, reflecting a thermodynamic control of V_{max} since the E_0' for the deaza analogue is 72 mV more negative than E_0' for riboflavin. Experiments using either 4(*R*)-[^{3}H]NADH (tritium in the transferable chiral locus) or ^3H_2O resulted in no incorporation of tritium at carbon-1 during aerobic catalysis involving multiple turnovers, consistent with an enolate structure for the dihydro-1-deazariboflavin anion. 1-DeazaFAD is an effective FAD analogue for apo-D-amino acid oxidase reconstitution and catalysis. Results are presented on binding of the coenzyme analogue and of inhibitors known to produce charge transfers with native holoenzyme. The V_{max} for turnover of several physiological D-amino acids by 1-deazaFAD-D-amino acid oxidase equals the V_{max} for native holoenzyme. The slow step in both instances is product release; the rate-determining physical step masks any effect the more negative redox potential of 1-deazaFAD might have on slowing down the coenzyme reduction step. Catalytic behavior of the 1-deazaFAD-enzyme with 3-haloamino acids, with nitroethane anion and with D-propargylglycine is qualitatively identical to the holoenzyme. 1-DeazaFAD also reconstitutes apoglucose oxidase from *Aspergillus niger*, functioning at a V_{max} = 0.10 that of FAD-glucose oxidase. Reduction of the coenzyme analogue is probably rate determining in turnover, with the lower redox potential of 1-deazaFAD controlling V_{max}. In agreement, [1-^{2}H]glucose shows a V_{max} kinetic isotope effect of 8.5.

Flavin-dependent enzymes catalyze many diverse and important redox transformations of organic and inorganic substrates in biological systems. These include flavoprotein dehydrogenases, many of which are membrane-bound components of electron-transport chains and are not reoxidized physiologically by molecular oxygen. The other broad category of flavoenzymes is one where the dihydroflavins are oxidized by molecular oxygen either functioning as oxidases, reducing O_2 to H_2O_2, or functioning as monooxygenases, activating O_2 for transfer of one oxygen atom to the substrate. Two important features of flavin chemistry relevant to its biological roles are its reduction potential and the reactivity of the dihydro and semiquinone forms for electron transfer to acceptors.

In the preceding paper of this issue we have probed some of the chemical and physical properties of a promising riboflavin analogue, 1-deazariboflavin.[1] In this manuscript we report

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‡ Present address: Department of Chemistry, Harvard University, Cambridge, Mass. 02138.

experiments on the redox behavior of 1-deazariboflavin and enzymatically prepared 1-deazaFAD with the NAD(P)H:flavin oxidoreductase, and with the homogeneous flavoprotein oxidases, D-amino acid oxidase and glucose oxidase. We have focused on the altered redox potential of 1-deazariboflavin (−280 mV vs. −208 mV for riboflavin) and its oxygen reactivity with these enzymes.

Experimental Procedure

Materials. 1-Deazariboflavin was the very generous gift of the synthetic chemistry group (Ashton et al., 1977) of Merck, Sharpe and Dohme Research Laboratories. This material was converted to 1-deazaFAD with the partially purified flavokinase and FAD synthetase from *Brevibacterium ammoniagenes* as described in Spencer et al. (1976) for 5-deazariboflavin.

The enzymes NAD(P)H:flavin oxidoreductase from *Beneckea harveyi,* D-amino acid:oxygen oxidoreductase (deaminating) from hog kidney (EC 1.4.3.3), and β-D-glucose:oxygen oxidoreductase from *Aspergillus niger* (EC 1.1.3.4) were prepared, resolved, and reconstituted as previously described (Fisher et al., 1976).

5-Deazariboflavin, D-erythro-3-chloro-2-aminobutyrate, D-[2-^{2}H]alanine, D,L-[2-^{3}H]alanine, D,L-3-bromoalanine, D,L-[2-^{3}H]proline, 4(*R*)-[^{2}H]NADH, and 4(*R*)-[^{3}H]NADH were synthesized and purified in this laboratory by published procedures (O'Brien et al., 1970; Spencer et al., 1976; Fisher et al., 1976; Walsh et al., 1973; Dang et al., 1976). D-3-Fluoroalanine was a gift of Dr. Janos Kollonitsch of Merck. All other materials were obtained from commercial sources.

Methods. The *B. harveyi* oxidoreductase was assayed by monitoring NADH oxidation at 340 nm at 30 °C in 0.1 M pyrophosphate, pH 8.6. Glucose oxidase was assayed by oxygen consumption in a Clark-type electrode chamber, volume 0.40 mL, at 30 °C in 0.1 M phosphate, pH 6.1. D-Amino acid oxidase was assayed in the Clark electrode or, for alanine and the substituted alanines, by NADH consumption at 340 nm in the presence of excess lactate dehydrogenase, with all assays at 30 °C in 0.1 M pyrophosphate, pH 8.3. A trace amount of catalase was added to all assays of the two oxidases.

The partitioning of chloroalanine between the oxidative and eliminative pathways in 1-deaza-D-amino acid oxidase was assayed by two independent methods: (1) Continuous assay for oxygen consumption in the Clark electrode, and continuous assay at 340 nm for total turnover with excess lactate dehydrogenase and NADH (chloropyruvate is a substrate for lactate dehydrogenase). The difference between these represents chloride elimination. (2) Discontinuous product assay, by preparation of the 2,4-dinitrophenylhydrazones of enzyme assay aliquots (Walsh et al., 1971), and comparison with the spectra of known mixtures of the hydrazone derivatives of pyruvate and chloropyruvate to determine the ratio of products. The two methods were in good agreement.

All other techniques were as previously described (Spencer et al., 1977; Fisher et al., 1976).

[1] Abbreviations used are: NADH, reduced nicotinamide adenine dinucleotide; EDTA, (ethylenedinitrilo)tetraacetic acid; 1-deazariboflavin and 1-deazaRF, 1-carba-1-deazariboflavin; 5-deazariboflavin and 5-deazaRF, 5-carba-5-deazariboflavin; 1-deazaFMN, 1-carba-1-deazariboflavin 5′-phosphate; 1dFAD and 1-deazaFAD, 1-carba-1-deazariboflavin 5′-diphosphate, 5′→5′-adenosine ester; 5-deazaFAD, 5-carba-5-deazariboflavin 5′-diphosphate, 5′→5′-adenosine ester. Two-electron-reduced neutral flavins are indicated as RFH_2, $FMNH_2$, and $FADH_2$, and the two-electron-reduced anions as RFH^-, $FMNH^-$, and $FADH^-$; these are preceded by 1-deaza- or 5-deaza- to indicate the appropriate two-electron-reduced analogue. "Natural" or "parent" flavin refers to unsubstituted riboflavin, FMN, or FAD; "native" enzyme or "holoenzyme" refers to enzyme as purified with its physiological coenzyme.

Results and Discussion

Preparation of 1-deazaFAD. Phosphorylation and adenylylation of any riboflavin analogue are prerequisite steps for its use as a coenzyme, and it is fortunate in this regard that 1-deazariboflavin is a substrate for the FAD-synthetase complex of *Brevibacterium ammoniagenes* (Spencer et al., 1976). This enzymatic conversion of 1-deazariboflavin to 1-deazaFAD (the accumulating product) occurs at a rate comparable to that for riboflavin to FAD and for 5-deazariboflavin to 5-deazaFAD. 1-DeazaFMN is then readily prepared by snake venom phosphodiesterase cleavage of 1-deazaFAD. The very limited quantities of 1-deazariboflavin available have precluded even the use of improved chemical phosphorylation procedures (Scola-Nagelschneider and Hemmerich, 1976), which, in any case, have neither the quantitative yield nor complete 5′-hydroxyl specificity of the enzymatic method (Spencer et al., 1976). The present work represents results obtained with approximately 10 μmol of 1-deazaFAD.

Both 1-deazaFMN and 1-deazaFAD are easily purified by Bio-Gel P2 adsorptive chromatography in water. 1-DeazaFAD in aqueous solution presumably has a conformation very similar to that of FAD, since, though it has no flavin fluorescence to be quenched by the adenine, it has, like FAD and 5-deazaFAD, an absorbance spectrum red shifted with loss of extinction (ϵ_{540} 6300 M^{-1} cm^{-1}) in the long-wavelength peak relative to 1-deazariboflavin or 1-deazaFMN (see Figure 1 of Spencer et al., 1977).

NAD(P)H:Flavin Oxidoreductase. In order to establish the biological redox activity of the 1-deazaflavins, this analogue was first examined as a substrate for the *Beneckea harveyi* NAD(P)H:flavin oxidoreductase. This enzyme, which was also the first used to examine the activity of 5-deazariboflavin (Fisher and Walsh, 1974; Fisher et al., 1976), offers the advantages of utilizing the flavin as a substrate rather than as a bound coenzyme and of accepting the flavin at the riboflavin, as well as FMN, level.

1-Deazariboflavin is competent in this enzyme catalysis (eq 1), with a V_{max} 0.025 that with riboflavin as substrate.

$$\text{1-deazaRF} + \text{NADH} \overset{\text{enzyme}}{\rightleftharpoons} \text{1-deazaRFH}^- + \text{NAD}^+ \qquad (1)$$
$$\text{1-deazaRF} + O_2$$

In an aerobic assay, no change is seen in the absorption spectrum of 1-deazariboflavin; however, under anaerobic conditions the 535-nm absorbance of 1-deazariboflavin is bleached through a series of isosbestic spectra identical to those observed in chemical reduction of 1-deazariboflavin by dithionite, hydrogen over platinum, or light/EDTA (Spencer et al., 1977). These spectra prove the identity of dihydro-1-deazariboflavin produced enzymatically with that produced in the cited chemical reductions. The possibility of a contaminating flavin acting as an intermediate electron carrier in these reactions is eliminated both by the high purity of the sample (no detectable isoalloxazine fluorescence) and the unique kinetic parameters of the reaction (Table I).

1-Deazariboflavin saturates the oxidoreductase at very low concentrations, having a K_m of 20 nM which is some 15-fold lower than that of riboflavin (K_m = 0.30 μM). In addition, 1-deazariboflavin is a competitive inhibitor of riboflavin reduction (K_i = 40 nM). In assay with 4(*R*)-[^{2}H]NADH, 1-deazariboflavin shows a kinetic isotope effect on V_{max} of 2.5, indicating that this analogue exhibits the same stereospecificity and partially rate-determining step as in enzyme turnover with riboflavin or 5-deazariboflavin.

TABLE I: Steady-State Kinetic Parameters for 1-Deazariboflavin Turnover with the *B. harveyi* NAD(P)H:Flavin Oxidoreductase.

	Riboflavin	1-Deazaribo-flavin
$V_{max,relative}$ with NADH	1.00	0.025
$V_{max,relative}$ with NADPH	0.80	0.084
$K_{m,flavin}$, with NADH (μM)	0.30	0.02
K_i vs. riboflavin and NADH (μM)		0.04
V_{max}, [^{1}H]NADH/V_{max}, (4*R*)-[^{2}H]NADH	4.8	2.5

These kinetic parameters are consistent with our expectations from the low redox potential of 1-deazariboflavin (E_0' = −280 mV; Spencer et al., 1977). With flavin reduction partially rate determining in riboflavin turnover (Fisher et al., 1976), substitution of 1-deazariboflavin, which is approximately 70 mV more difficult to reduce, should both dramatically lower V_{max} and preserve flavin reduction as at least partially rate determining; both of these expectations are clearly met (Table I). The low K_m and K_i of 1-deazariboflavin are probable consequences of a large decrease in V_{max} with relatively small changes in the substrate binding and release rate constants.[2] Also consistent with the value of the redox potential of 1-deazariboflavin is the observation of enzyme-catalyzed equilibration of NADH and 1-deazariboflavin under anaerobic conditions, K_{eq} = 22 ± 7 (Spencer et al., 1977).

The importance of the enzymatic orientation of the two substrates at the oxidoreductase active site is emphasized by the nicotinamide specificity of the enzyme with 1-deazariboflavin. Where NADPH is an equally potent reductant of riboflavin (relative V_{max} = 0.8 that with NADH) but at least a tenfold poorer one for 5-deazariboflavin, the V_{max} observed for NADPH oxidation with 1-deazariboflavin is 3.5-fold higher than that of NADH.

We have also used the oxidoreductase to investigate the fate of the substrate-derived hydrogen transferred during catalysis. The nonenzymatic studies with 1-deazariboflavin have shown that the hydrogen at C-1 of oxidized 1-deazariboflavin is stable, and that this same hydrogen of dihydro-1-deazariboflavin only slowly exchanges with solvent protons (Spencer et al., 1977). The two suitable chemical reduction procedures (dithionite and EDTA photoreduction) have as their ultimate hydrogen source the solvent protons; thus, these chemical studies are not a model for direct hydrogen transfer to the C-1 position of 1-deazaflavins.[3] In the oxidoreductase-catalyzed reduction of 5-deazariboflavin by NADH, hydrogen is transferred directly and completely from the 4*R* position of the NADH to C-5 of the 5-deazariboflavin (Fisher and Walsh, 1974; Fisher et al., 1976), implying that the substrate carbon–hydrogen bond being cleaved is very probably in close proximity to position 5 of the flavin or flavin analogue being reduced (Blankenhorn, 1976). Nonetheless, to assay for possible incorporation of substrate-derived hydrogen at position 1, 1-deazariboflavin was incubated with a several equivalent excess of 4(*R*)-[^{3}H]NADH and oxidoreductase under aerobic conditions. While C-1 labeled dihydro-1-deazaflavins do exchange with solvent, this exchange occurs much more slowly than the competing oxygen oxidation (Spencer et al., 1977), and any tritium originating from NADH, if it is indeed transferred to C-1, should be irreversibly locked into this position in the oxidized flavin. The results from the experiment demonstrated complete loss of the label from the 4*R* position of NADH to solvent, with *no* detectable incorporation of tritium label into the recovered oxidized 1-deazariboflavin. The complementary experiment, NADH reduction of 1-deazariboflavin in ^3H_2O, also resulted in recovery of completely nonradioactive oxidized 1-deazariboflavin. These results are best interpreted by the retention of the 5 position of the 1-deazaflavin as the locus for the hydrogen transferred from NADH, with the dihydro-1-deazaflavin thus formed capable of only slow solvent exchange at C-1.

D-Amino Acid Oxidase. Most flavoenzymes bind the flavin cofactor as a tightly bound coenzyme. To pursue flavin-analogue studies with such an enzyme, one must have large amounts of highly purified enzyme available and have an efficient procedure for the *reversible* removal of the flavin (for replacement by the analogue). D-Amino acid oxidase from hog kidney meets both these criteria, and in addition has produced a detailed chemical and kinetic mechanistic literature (Walsh et al., 1971, 1973; Porter and Bright, 1976; Cheung and Walsh, 1976; Porter et al., 1977). For these reasons, we have focused our greatest attention on 1-deazaFAD-substituted D-amino acid oxidase, and will discuss: (1) its static properties of coenzyme analogue and inhibitor binding, (2) its turnover of physiological substrates, (3) isotope kinetic and incorporation experiments, and (4) turnover of substrate analogues, specifically 3-haloamino acids and nitroalkane anions.

1-DeazaFAD Binding. The binding of 1-deazaFAD to apo-D-amino acid oxidase results in a slight drop in the long-wavelength extinction coefficient of the coenzyme analogue (to $\epsilon_{540} \simeq 5900\ M^{-1}\ cm^{-1}$) without shouldering or vibronic resolution, paralleling the absence of spectral perturbations on binding of FAD or 5-deazaFAD to this apoenzyme (Massey and Curti, 1966; Fisher et al., 1976; Hersh and Jorns, 1975). Since 1-deazaFAD is nonfluorescent, flavin fluorescence quenching cannot be used to assay binding to the apoenzyme, but an indirect assay is available in the FAD fluorescence increase realized on the equilibration of FAD–enzyme with exogenous 1-deazaFAD. This coenzyme binding-competition assay gives a K_D for 1-deazaFAD of 5.6 ± 1.3 μM (Table II) corresponding to a binding of 1-deazaFAD some 24-fold poorer than FAD.

An additional measure of flavin affinity is the apparent K_m of the coenzyme in a turnover assay. In D-alanine oxidation, apoenzyme exhibits saturation kinetics in coenzyme with both FAD and 1-deazaFAD, with the result (Table II) that the apparent K_m of 1-deazaFAD is 15-fold higher than that of FAD. Thus, the poorer binding of the 1-deazaFAD is also evident in a kinetic assay. In addition, since the K_m for either coenzyme is three- to fivefold *lower* than its static K_D, a binary enzyme–flavin complex cannot be the major steady-state species in turnover. Instead, the predominant form of the enzyme (with *either* coenzyme) must be a ternary complex of enzyme–flavin–substrate or enzyme–flavin–product, and this ternary species must bind the flavin more tightly than the apoenzyme alone. This is suggestive of a common rate-determining step in turnover for both FAD and 1-deazaFAD, as will be discussed more directly below.

Benzoate Binding. Titration of 1-deazaFAD–D-amino acid oxidase with sodium benzoate results in dramatic vibronic resolution of the bound 1-deazaFAD (Figure 1), qualitatively

[2] Though the kinetic mechanism of the oxidoreductase is not known sufficiently well to couch the relation between k_{cat} and $K_{m,Fl}$ in explicit rate constants, probably all that is required for a positive correlation between these two observables is that k_{cat} exceed the rate constant for the dissociation of oxidized flavin. The direct relationship between V_{max} and $K_{m,Fl}$ is remarkably apparent in the data obtained with a number of enzymatically active flavin isosteres (Walsh et al., 1977).

[3] In contrast to 5-deazaflavin model studies, in which sodium borohydride reduction proceeds by direct hydrogen transfer to C-5 (Spencer et al., 1976).

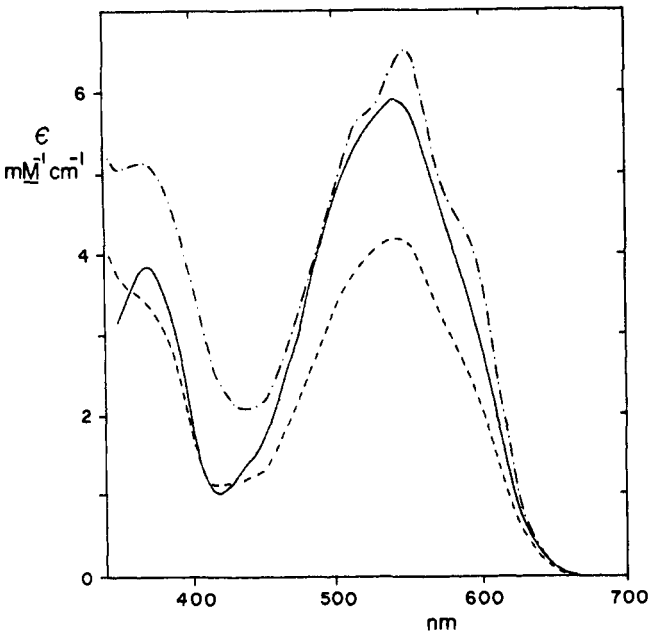


FIGURE 1: (—) 1-deazaFAD-D-amino acid oxidase in 0.1 M KPP_i, pH 8.3, + (- - -) sodium sulfite (pH 8.3) to 0.31 M, + (- · -) sodium benzoate to 3.3 mM. The spectrum was identical to that obtained on benzoate addition to sulfite-free 1-deazaFAD-enzyme; initial $A_{540} = 0.142$.

TABLE II: Static Parameters for Association with D-Amino Acid Oxidase.[a]

K	FAD	1-deaza-FAD
K_D, coenzyme (μM)	0.23 ± 0.08	5.6 ± 1.3
Apparent K_m for the coenzyme in D-alanine turnover[b] (μM)	0.082	1.2
K_D, benzoate (μM)	3[c]	100
K_D, sulfite		
Free coenzyme (M)	1	16
D-Amino acid oxidase (mM)	3.4[d]	900 m*M*

[a] Obtained at 30 °C in pyrophosphate, pH 8.3, buffer. [b] 56 mM in D-alanine. [c] Massey and Ganther (1965). [d] Massey et al. (1969).

similar to that observed in benzoate-FAD-enzyme (Massey and Ganther, 1965) and benzoate-5-deazaFAD-enzyme (Hersh and Jorns, 1975; Fisher et al., 1976). Such titration gives a K_D for benzoate of 100 μM, some 30-fold higher than that for FAD-enzyme (Table II). However, benzoate competitively inhibits oxidation of D-alanine by 1-deazaFAD-enzyme with a K_i of approximately 6 μM. This difference between the apparent K_D and K_i suggests that in the static titration experiment a significant fraction of the enzyme was dissociated from its coenzyme (eq 2), and thus the apparent

$$\begin{array}{ccccc} \text{E} + \text{1dFAD} & \overset{K_{D,F}}{\rightleftharpoons} & \text{E-1dFAD} & \overset{S}{\rightleftharpoons} & \text{E-1dFAD-S} \\ & & \overset{B}{\Big\Vert} K_{D,B} & & \Big\downarrow \text{slow} \\ & & \text{E-1dFAD-B} & & \text{E-1dFAD} + \text{P} \end{array} \quad (2)$$

K_D for benzoate is a function of the true K_D (the dissociation constant for E-1dFAD + B ⇌ E-1dFAD-B) and the K_D of the coenzyme. In the kinetic experiment, the enzyme exists largely as a ternary complex (vide supra), with the binary E-1dFAD a minority species that is rapidly complexed with substrate or the inhibitor benzoate; thus the low K_i for benzoate is a better measure of the binding of benzoate than the static K_D, since

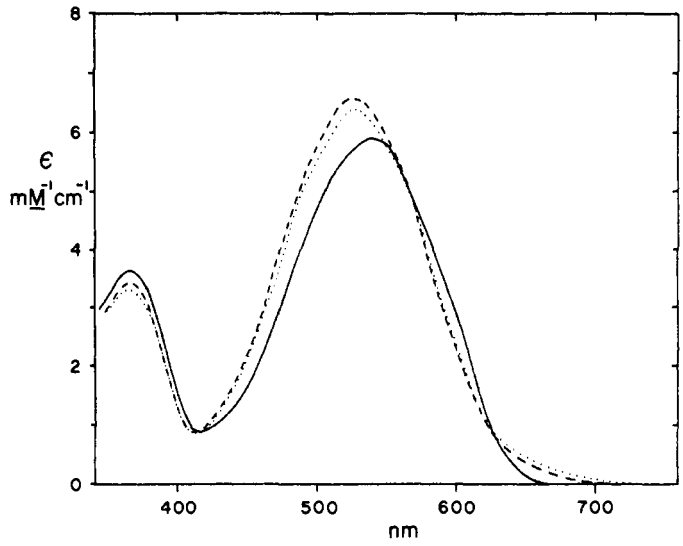


FIGURE 2: (—) 1-deazaFAD-D-amino acid oxidase in 0.1 M KPP_i, pH 8.3; (· · ·) 5 min at 20 °C after addition of D-3-chloro-2-aminobutyrate to 0.82 mM. (- - -) Separate experiment: 1-deazaFAD-D-amino acid oxidase plus sodium anthranilate to 1.7 mM; initial $A_{540} = 0.108$.

the enzyme and coenzyme remain associated during turnover.[4]

Anthranilate Binding. The spectral shifts on binding of sodium anthranilate to 1-deazaFAD-enzyme are much less pronounced than those of its binding to FAD-enzyme. In the 1-deazaFAD case, the long-wavelength peak blue shifts and increases in extinction (to $\epsilon_{523} \simeq 6600\ M^{-1}\ cm^{-1}$) and a tailing of this peak appears between 620 and 740 nm (Figure 2). Unlike the FAD-enzyme-anthranilate complex (Massey and Ganther, 1965), there is no distinct, long-wavelength charge-transfer band. There may be charge-transfer character in the 1-deazaFAD-enzyme-anthranilate complex, but it is obscured by the 1-deazaFAD transition at 540 nm, with only the 620- to 740-nm absorption tail betraying its existence. In any case, the spectrum of the complex is distinct from that of 1-deazaFAD-enzyme alone, and is thus of use in the interpretation of the spectra observed during turnover of 3-chloro-2-aminobutyrate (vide infra).

Sulfite Addition. Sulfite adds to the electrophilic N-5 of oxidized flavins to yield adducts with spectra similar to those of dihydroflavins (Müller and Massey, 1969). The binding of FAD to apo-D-amino acid oxidase increases the affinity of the flavin for sulfite by a factor of nearly 300 (Table II); this increased electrophilicity of the bound flavin may be a direct reflection of its E_0', raised 200 mV on binding (Brunori et al., 1971; Jorns and Hersh, 1976). An excellent correlation between E_0' and K_D for sulfite of free flavins is well established (Müller and Massey, 1969).

In this vein, we find that the affinity of 1-deazaFAD for sulfite is similarly increased on coenzyme binding to apo-D-amino acid oxidase, with the K_D for sulfite addition decreasing from 16 to 0.9 M. The sulfite-1-deazaFAD-enzyme adduct is bleached at 540 nm, and in these experiments is indistinguishable from the spectrum of dihydro-1-deazaflavins (Figure 1). The value of 0.9 M is an upper limit to the sulfite-1dFAD-enzyme K_D, due to a possible perturbation from coenzyme-apoenzyme dissociation, as discussed with respect to benzoate binding. As is the case for the sulfite-FAD-enzyme adduct, the addition of excess benzoate rapidly displaces

[4] At the concentrations used, substrate binding to 1-deazaFAD enzyme (by analogy to native enzyme, Porter et al., 1976) is likely to be much faster than 1-deazaFAD dissociation.

the sulfite equilibrium to yield the spectrum of the oxidized 1-deazaFAD–enzyme–benzoate complex (Figure 1).

Binding of Dihydro-1-DeazaFAD; Redox Potential. The increased affinity for sulfite of 1-deazaFAD bound to D-amino acid oxidase over free 1-deazaflavin indicates that the enzyme-bound 1-deazaflavin has a higher redox potential than the free analogue (vide supra; also Jorns and Hersh, 1976). This change in redox potential could be as much as 100 mV.[5]

An increased redox potential of a flavin when enzyme bound is a consequence of the apoenzyme binding the reduced coenzyme more tightly than the oxidized. We therefore looked for a spectroscopic indication of dihydro-1-deazaFAD binding to apo-D-amino acid oxidase, particularly since dramatic spectral changes on binding of dihydro-1-deazariboflavin to the egg-white binding protein are seen (Spencer et al., 1977). No such changes are seen on binding of dihydro-1-deazaFAD by apo-D-amino acid oxidase; in fact, the absorbance tail of the dihydro-1-deazaflavin above 400 nm is decreased, indicating a drop in the pK_a of dihydro-1-deazaFAD of approximately 0.5 pH unit to a value of approximately 5.1.

The differences in binding of dihydro-1-deazaflavins by the egg-white flavin-binding protein and apo-D-amino acid oxidase deserve comment. As is evident from the data, the two apoproteins stabilize different charge forms of the dihydro-1-deazaflavin. The anionic form bound to apo-D-amino acid oxidase has a structure similar to that preferred in free aqueous solution (the several relevant tautomers and resonance forms of dihydro-1-deazaflavins are discussed in Spencer et al., 1977). We suggest that the differences in the dihydro-1-deazaflavin forms stabilized by these two proteins are characteristic of dehydrogenase-type flavin sites (the binding protein) and oxidase-type sites (D-amino acid oxidase), and thus the readily measured spectrum and pK_a of dihydro-1-deazaflavin bound to a given apoprotein may characterize that protein. Other probes that correlate with the oxygen reactivity of flavoproteins are the absorption spectrum and ESR line width of the preferred flavin radical (Massey et al., 1969; Palmer et al., 1971), and the reactivity with sulfite (Massey et al., 1969).

Catalytic Competence; D-Alanine Steady-State Kinetics. The addition of D-alanine to an anaerobic solution of 1-deazaFAD-reconstituted D-amino acid oxidase results in the rapid and near complete bleaching of the chromophore. The spectra during the course of 1-deazaFAD reduction are essentially identical to those observed during the reduction of 1-deazariboflavin by dithionite (Spencer et al., 1977) or by NADH and the *B. harveyi* oxidoreductase. Since dihydro-1-deazaflavins are rapidly oxidized by molecular oxygen, it is expected, and found, that 1-deazaFAD–D-amino acid oxidase is catalytically competent in the oxidation of several D-amino acids. The kinetic parameters for these substrates are listed in Table III, along with the values determined with FAD-reconstituted apoenzyme. The 1-deazaFAD enzyme evidently retains the substrate specificity and catalytic efficiency of the native enzyme, despite its low redox potential and poorer binding. Though adequately reproducible, the K_m values in Table III for 1-deazaFAD–enzyme may represent upper limits, due to the coenzyme binding considerations discussed with respect to benzoate affinity (vide supra). That 1-deazaFAD- and FAD–enzymes have *fully comparable* V_{max} values for these substrates suggest that both forms of the enzyme have a common rate-determining step, and that step is *not critically dependent on the flavin redox chemistry.* Since product imine release is clearly rate determining for FAD–enzyme (for D-alanine it is 400-fold slower than flavin reduction, Porter et al., 1977), we propose that this is also the case for 1-deazaFAD–enzyme.

TABLE III: Steady-State Kinetic Parameters for D-Amino Acid Oxidase.[a]

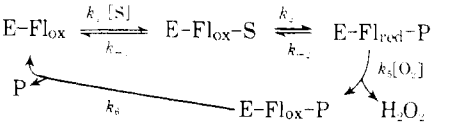
	FAD		1-deazaFAD	
	V_{max} (s^{-1})	K_m (mM)	V_{max} (s^{-1})	K_m (mM)
D-Proline	40	2	23	5
D-Methionine	11	0.3	10	0.6
D-Alanine	9	0.6	9	3.3
D-[2-^{2}H]Alanine	9	0.6	8	3.3
D-Serine	5	2	4	90
D-Glutamate	0		0	
Oxygen, saturating D-alanine		0.16		0.015
3-Chloro-D-alanine	2.5	0.5	0.9	3
3-Chloro-D-2-aminobutyrate	0.2[b]	0.5	0.4[b]	3

[a] Obtained at 30 °C in air-equilibrated 0.10 M pyrophosphate, pH 8.3, buffer. [b] Independent of oxygen concentration.

The steady-state kinetics of oxidation of D-alanine by 1-deazaFAD–enzyme were pursued in greater detail. With excess NADH and lactate dehydrogenase in the oxygen-electrode chamber to remove inhibition by pyruvate, plots of $[O_2]$ vs. time can be applied to the integrated rate equation to yield the V_{max} and K_m with respect to oxygen in single enzymatic runs (Bright and Porter, 1975). From such assays with several different concentrations of D-alanine, it was observed that 1-deazaFAD–D-amino acid oxidase exhibits parallel-line kinetics. Native enzyme also shows parallel-line kinetics with D-alanine, though in mechanism it is unambiguously ordered sequential and *not* ping pong. The reason for this apparent discrepancy is that the ternary term has a coefficient (ϕ_{12}, eq 3) that is vanishingly small relative to the other terms, as has recently been quantitated by Porter et al. (1977).

$$\frac{[E_{tot}]}{V} = \phi_0 + \frac{\phi_1}{[S]} + \frac{\phi_2}{[O_2]} + \frac{\phi_{12}}{[S][O_2]} \qquad (3)$$

The parallel-line kinetics and V_{max} values of 1-deazaFAD–enzyme suggest that the minimal kinetic scheme for the native enzyme of Porter et al. (1977) applies to the analogue-substituted enzyme as well (eq 4). Of particular note is the low K_m for oxygen of 1-deazaFAD–enzyme (Table III); thus, the apparent bimolecular rate constant for oxygen oxidation of reduced 1-deazaFAD–enzyme ($\phi_2^{-1} = V_{max}/K_{m,O_2}$) is tenfold higher than that of FAD–enzyme. The simplest explanation for this difference is that the elementary rate constant for oxygen reaction (k_5; eq 4) is tenfold higher for 1-deazaFAD enzyme than for FAD enzyme.



(4)

This is not unreasonable since the oxygen oxidation of dihydro-1-deazariboflavin in neutral aqueous solution is twofold faster than that of dihydroriboflavin. Cytochrome *c* reduction assays show no detectable superoxide production by 1-deazaFAD–D-amino acid oxidase during the oxidation of D-alanine under conditions that would have easily detected 0.01 mol of

[5] Inferred from the change in FAD redox potential on enzyme binding, and assuming that other contributions to sulfite affinity (electrostatic interactions of active-site residues, solvent exclusion, etc.) remain the same for FAD and 1-deazaFAD enzyme.

superoxide per mol of D-alanine oxidized. Thus, as is also true of native enzyme, any superoxide formed in the oxidation of enzyme-bound dihydro-1-deazaFAD undergoes exclusive radical recombination with 1-deazaFAD semiquinone followed by heterolytic bond cleavage to form hydrogen peroxide.

Isotope Substitution: Steady-State Kinetics. Native D-amino acid oxidase shows no isotope effect at V_{max} in the oxidation of D-[2-^{2}H]alanine; in rapid-kinetics studies, however, an isotope effect of approximately 4 is seen on FAD reduction as monitored at 450 nm (Porter et al., 1977). The absence of an isotope effect on the steady-state turnover is due to the rate-determining product release being much slower than the FAD reduction (400-fold slower with D-[2-^{1}H]alanine, 100-fold slower with D-[2-^{2}H]alanine; Porter et al., 1977). We reasoned that 1-deazaFAD, with its low redox potential, might be sufficiently slow in the reductive half-reaction such that 1-deazaflavin reduction might approach product release as the slow step in catalysis. If this were the case with D-[2-^{2}H]alanine as substrate, 1-deazaFAD-enzyme might show a steady-state isotope effect on V_{max}. In addition, D,L-[2-^{3}H]-alanine as substrate could be used to measure the tritium-isotope effect, which is of necessity an effect on V_{max}/K_m.

The deuterium-isotope effects observed on V_{max} are 1.2 ± 0.1 for 1-deazaFAD-enzyme, and 1.1 ± 0.05 for FAD-enzyme; on V_{max}/K_m, 1.3 ± 0.1 for 1-deazaFAD-enzyme and 1.1 ± 0.1 for FAD-enzyme. The tritium-isotope effects on V_{max}/K_m are 1.3 ± 0.1 for 1-deazaFAD and 1.3 ± 0.05 for FAD-enzymes. There are three reasons why 1-deazaFAD-enzyme could show low isotope effects that are only marginally higher than the FAD-enzyme controls: (1) Substrate C-H bond scission may indeed be slowed in 1-deazaFAD-enzyme catalysis, but this decrease is less than a factor of 100, such that product release remains rate determining, thereby masking the 1-deazaFAD reduction isotope effect. (2) The low redox potential of 1-deazaFAD does cause C-H bond scission to become significantly rate determining, but at the same time this low redox potential makes the reverse redox reaction (k_{-2}, eq 4) kinetically significant, thereby reducing the isotope effect to an equilibrium effect. The elegant work of Porter et al. (1977) demonstrates that the α proton of phenylalanine is conserved during FAD-enzyme catalysis, and that the deuterium-isotope effect on k_{-2} is comparable to that on k_2. (3) The transition state of k_2 for 1-deazaFAD-enzyme may be significantly more asymmetric than that of FAD-enzyme, such that the isotope effect on k_2, and thus on V_{max}, is intrinsically low. Of these possibilities, we favor 1, since our other evidence suggests that it is still product release that is rate determining in 1-deazaFAD-enzyme catalysis.

Isotope Substitution: Label Incorporation. As with the *B. harveyi* oxidoreductase (vide supra), it is of mechanistic importance to determine whether hydrogen derived from substrate and/or solvent becomes stably incorporated at C-1 of 1-deazaFAD-D-amino acid oxidase during catalysis. Studies with 5-deazaFAD-D-amino acid oxidase have demonstrated direct and complete hydrogen transfer from C-2 of the substrate to C-5 of the bound coenzyme (Hersh and Jorns, 1975; Fisher et al., 1976). These loci are likely in very close proximity, a relationship also expected for the substrate C-2 and coenzyme N-5 of both FAD- and 1-deazaFAD-enzyme.

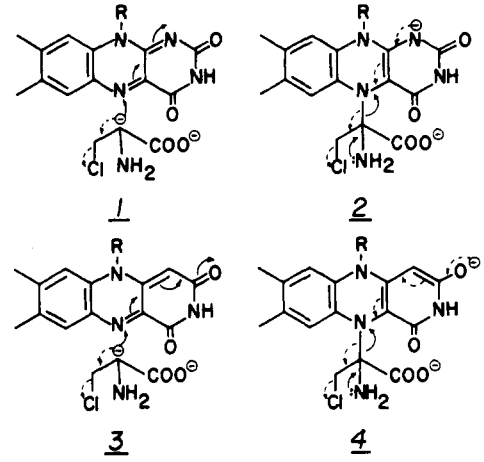
Consistent with this are our findings with 1-deazaFAD-enzyme: incubation of 17 nmol of 1-deazaFAD-enzyme with 350 nmol of D,L-[2-^{3}H]proline for 5 min at 20 °C resulted in release of 53% of the label to solvent, with the remaining 47% entirely accounted for in unreactive L-[2-^{3}H]proline. The isolated 1-deazaFAD showed absolutely no radioactivity. As assay for possible acquisition of solvent protons during catalysis, 12 nmol of 1-deazaFAD-enzyme was incubated with 25 μmol of D-alanine in 0.43 mL of ^3H_2O (4.2 Ci/mol; 70 mM KPP_i, pH 8.3). The initially air-saturated solution was allowed to go anaerobic, and the reaction was then terminated by the addition of trichloroacetic acid to denature the enzyme.[6] Tritiated water was removed by lyophilization and the 1-deazaFAD isolated to yield a completely nonradioactive coenzyme. This finding is consistent with the result from the comparable oxidoreductase experiment and with an enolate structure for the dihydro-1-deazaFAD bound to apo-D-amino acid oxidase (structure 5b or 5d of Spencer et al., 1977).

TABLE IV: D-Amino Acid Oxidase: Partitioning of Chloroalanine between Oxidation and Elimination.

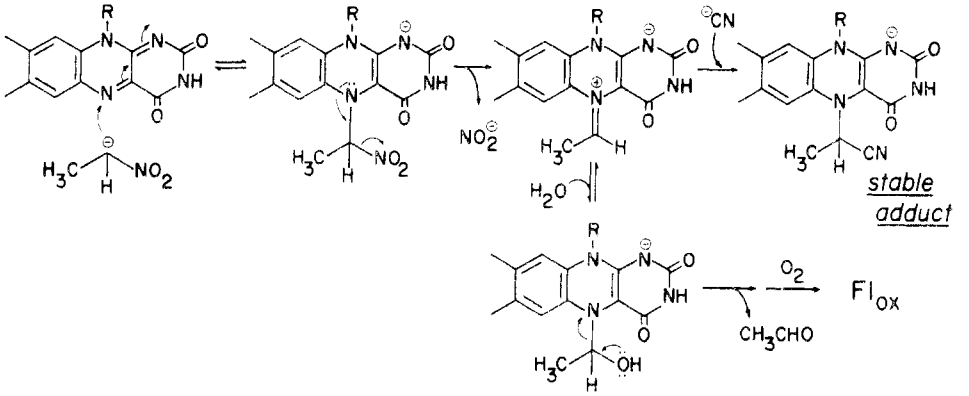
	Coenzyme			
	FAD[a]		1-deazaFAD	
Atm	Oxidn (%)	Elimination (%)	Oxidn (%)	Elimination (%)
Argon	0	100	0	100
Air	65	35	30	70
Oxygen	95	5	50	50

[a] Walsh et al. (1971).

Turnover of Halogenated Substrates. 3-Halogenated amino acids have proven to be substrates for D-amino acid oxidase of particular mechanistic interest (Walsh et al., 1971, 1973; Porter and Bright, 1976; Dang et al., 1976). 3-Chloro-D-alanine undergoes both enzyme-catalyzed oxidation and elimination; the partition between these pathways is a function of the oxygen concentration. One of the major unsolved questions in D-amino acid oxidase catalysis is the structure of the partitioning species. Two viable alternatives for this species are the carbanion **1** and the dihydroflavin N-5-adduct **2**. The oxidative pathway for each is shown by solid arrows, and the eliminative by dashed arrows. Recent experiments have been interpreted as favoring **1** (Dang et al., 1976), and alternatively **2** (Bright and Porter, 1975; Massey et al., 1976). We have investigated the turnover of several 3-halogenated amino acids by 1-deazaFAD-D-amino acid oxidase in an attempt to resolve this question.



[6] It is conceivable that solvent hydrogen *is* acquired at C-1 on bound 1-deazaFAD reduction, but that on coenzyme oxidation this same prochiral hydrogen is stereospecifically returned to solvent. Denaturation of the enzyme while anaerobic and the subsequent nonenzymatic oxidation of the dihydro-1-deazaFAD provide at least one turnover of achiral dihydro-1-deazaflavin oxidation; with the high sensitivity of the radioassay, we are thus assured that solvent hydrogen is *never* incorporated at C-1, even as a chiral enzyme-bound intermediate in catalysis.



(5)

The results of these experiments are presented in Tables III and IV. 1-DeazaFAD-enzyme behaves in much the same manner as FAD-enzyme, partitioning chloroalanine between oxidation (to chloropyruvate) and elimination (to pyruvate) as a function of oxygen concentration, with, again, the sum of the two pathways giving a constant V_{max} independent of oxygen concentration. The parallels with the FAD-enzyme behavior continue in that there is complete elimination of halide from both bromoalanine and 3-chloro-2-aminobutyrate, without any consumption of oxygen, and in the complete (>95%) oxidation of fluoroalanine.

The notable difference between FAD and 1-deazaFAD enzymes in these experiments is the finding that 1-deazaFAD-enzyme preferentially eliminates chloride from chloroalanine (Table IV). Unfortunately, this observation, while fully consistent with the known chemistries of flavins and 1-deazaflavins, does not allow us to distinguish between the two partitioning intermediate candidates, as follows. (1) The low redox potential of 1-deazaFAD, by making the oxidized coenzyme analogue more difficult to reduce than FAD, could be expected to increase the lifetime of the substrate carbanionic species **3**, thereby favoring elimination over oxidation. (2) If the adduct **4** were formed, the low redox potential of the 1-deazaFAD, by making its dihydro form a better reductant (more electron rich) than dihydroFAD, would again favor chloride elimination over substrate oxidation. In any case, the low potential of 1-deazaFAD is likely to alter the several rate constants involving coenzyme oxidoreduction considerably, so that, while our steady-state data do not distinguish between the two pathways, rapid kinetic studies with 1-deazaFAD-enzyme may resolve the uncertainty.

Elimination from 3-Chloro-2-aminobutyrate: Spectrum. A second use of halogenated substrates is the evidence their turnover may provide as to the rate-determining step in catalysis. Since 3-chloro-2-aminobutyrate and bromoalanine eliminate halide without consumption of oxygen, the steady-state spectrum of the enzyme in turnover of these substrates is easily observed. 3-Chloro-2-aminobutyrate, with its low turnover number, is particularly amenable to this assay. The steady-state spectrum of 1-deazaFAD-D-amino acid oxidase in 3-chloro-2-aminobutyrate turnover is shown in Figure 2. This spectrum developed within 2 min of addition of substrate, and persisted at ambient temperature for 2 h. There is a large similarity between this spectrum and that of the 1-deazaFAD-enzyme-anthranilate complex (also in Figure 2). Both show increased extinction of the long-wavelength peak with a 13- to 18-nm blue shift and a long-wavelength absorption from 620 to 740 nm. This close correspondence of spectra is also seen with FAD-enzyme, and is due to an E-Fl_{ox}···P complex of oxidized flavin with the eneamine (the elimination product of 3-chloro-2-aminobutyrate) (Cheung and Walsh, 1976) or the product analogue anthranilate (Walsh et al., 1973). Thus, for 1-deazaFAD-enzyme as well as native enzyme, it is an E_{ox}···P complex that accumulates in 3-chloro-2-aminobutyrate eliminative turnover, and thus with both coenzymes the rate-determining step is late in the mechanism, being either product release or an enzyme base deprotonation and conformational change concomitant with product release (Massey et al., 1976; Cheung and Walsh, 1976). This finding, with our observations on V_{max} (Table III) and the low V_{max} isotope effects, is conclusive evidence that product release is rate determining in catalysis by 1-deazaFAD-D-amino acid oxidase.

Reaction with Nitroethane Carbanion: Trap of the Imine-1-Deazaflavin Intermediate. The work of Porter et al. (1974) demonstrated the mechanistic utility of nitroalkane anions as preformed carbanionic substrates for D-amino acid oxidase. Through the use of nucleophilic trapping agents (borohydride, cyanide, etc.) they were able to intercept a kinetically invisible covalent substrate-flavin adduct (eq 5). The following observations suggest once more that 1-deazaFAD-enzyme catalyzes the same reactions as native enzyme.

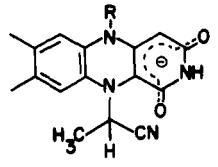
In the oxygen electrode, 1-deazaFAD-enzyme consumes oxygen on addition of nitroethane anion with a turnover number of approximately 0.002 s^{-1} (compared to 0.5 s^{-1} for FAD-enzyme). Addition of cyanide to 5 mM stops oxygen consumption within 2 s. In an anaerobic spectrophotometric assay, the nitroethane anion bleaches the 540-nm peak of bound 1-deazaFAD. After 70 min of such an anaerobic incubation, a withdrawn aliquot showed retention of 82% of the initial activity of the enzyme (for D-alanine oxidation), and, when the sample was aerated, the 540-nm absorbance of the 1-deazaflavin returned. In an aerobic spectrophotometric assay in 7 mM cyanide, addition of nitroethane anion to 1-deazaFAD-enzyme results in irreversible bleaching of the 540-nm absorbance, and assay for D-alanine oxidation showed 2.5% of the initial enzyme activity present when 87% of the chromophore was bleached. However, when exogenous 1-deazaFAD was added to the D-alanine oxidation assay, the activity increased to 53% of that initially, demonstrating that this inactivation proceeds by a modification of the 1-deazaflavin and not the apoenzyme. The spectra during such inactivation show that, while the 1-deazaFAD is bleached at 540 nm, it is distinct from dihydro-1-deazaflavins. The latter have isosbestic points at ca. 350, 400, and 410 nm at this pH, while the cyanide-nitroethane anion product spectra cross at ca. 360 nm but not above this wavelength. By analogy with FAD-enzyme, but without other structural evidence, we propose that this product is the 5-(2-cyanopropyl)dihydro-1-deazaflavin adduct **5**.

Alkylation by D-Propargylglycine. Horiike et al. (1975) first showed that D-amino acid oxidase is modified during oxidation of D-propargylglycine (D-2-amino-4-pentynoate). This modification does not alter the coenzyme but is an alkylation of the apoenzyme to produce a "wounded" enzyme,

TABLE V: Glucose Oxidase: Turnover Parameters.

	Coenzyme			
	FAD substrate		1-deazaFAD substrate	
	Glucose	[1-^{2}H]Glucose	Glucose	[1-^{2}H]Glucose
V_{max}[a] (s^{-1})	1400[b]	180	170	20
K_m, glucose[c] (mM)	44	26	54	20
K_m, oxygen[d] (μM)	330	74	26	7.5

[a] Turnover number per flavin. [b] From isotope effect of Bright and Gibson and V_{max} with [1-^{2}H]glucose; measured value $1500 \pm 500\ s^{-1}$ due to uncertainty at glucose concentrations >0.1 M. [c] Initial rate data under air saturation. [d] At 250 mM glucose, from complete oxygen consumption runs and fit to the integrated rate equation.



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competent in substrate oxidation but with drastically altered kinetics (Marcotte and Walsh, 1976; Marcotte and Walsh, in preparation).

In preliminary experiments, 1-deazaFAD-D-amino acid oxidase was alkylated 50-fold more efficiently than FAD-enzyme, corresponding to protein alkylation after approximately 13 turnovers as compared to 600 turnovers with holoenzyme (15 mM D-propargylglycine). The initial turnover numbers of the two enzymes were 0.13 and 1.8 s^{-1}, respectively. Whether this increased efficiency for alkylation with the 1-deaza coenzyme is a function of changed coenzyme chemistry or simply slightly altered ternary complex active-site geometry is not yet apparent and is under further investigation.

Glucose Oxidase. Like D-amino acid oxidase from hog kidney, glucose oxidase from *Aspergillus niger* meets the criteria for worthwhile substitution with coenzyme analogues. The annoying problem in the study of substituted glucose oxidase is the unavoidable presence of holoenzyme in apoenzyme preparations that have been subjected to as many as four cycles of acid–ammonium sulfate precipitation. Various apoenzyme preparations in our hands have had from 3 to 10% of the total (FAD) reconstitutable activity present as holoenzyme. This contamination notwithstanding, our kinetic findings with 1-deazaFAD-glucose oxidase are reproducible, dependent on added 1-deazaFAD, and kinetically distinct from FAD-glucose oxidase.

1-DeazaFAD bound to apoglucose oxidase shows an increase in its long-wavelength absorbance (to $\epsilon_{540} \simeq 7500\ M^{-1}\ cm^{-1}$) without vibronic resolution, similar to the behavior of FAD on binding. An anaerobic solution of 1-deazaFAD-glucose oxidase is rapidly bleached at 540 nm on addition of glucose to approximately 80% reduction. This extent of reduction is comparable to the maximum observed with native enzyme or 5-deazaFAD-enzyme (Fisher et al., 1976). The absorbance at 540 nm returns on aeration of the enzyme. The results of steady-state kinetic experiments in the oxygen electrode are presented in Table V, for FAD- and 1-deazaFAD-glucose oxidase reconstituted from the same apoenzyme. The reconstituted FAD-enzyme data are in reasonable agreement with previous work (Bright and Gibson, 1967).

Rapid kinetic studies with native enzyme have shown that three steps in catalysis (flavin reduction, lactone release, and peroxide release) can be fully or partially rate determining, depending on substrate deuteration, temperature, pH, and halide concentration (Bright and Gibson, 1967; Weibel and Bright, 1971). With V_{max} so "finely tuned", one might expect that the substitution of a coenzyme analogue would alter the reaction kinetics dramatically. Specifically, the use of 1-deazaFAD as the coenzyme should selectively slow coenzyme reduction (since the redox potential of 1-deazaflavins is 70 mV below that of flavins), and, unless the active-site geometry were significantly perturbed, have little effect on the physical steps of product release. That this simple view of coenzyme substitution may have some validity is shown by ease with which it accounts for the general kinetic patterns of 1-deazaFAD-glucose oxidase catalysis.

First, the V_{max} of 1-deazaFAD-glucose oxidase is an order of magnitude lower than that of the FAD-enzyme, implying that the coenzyme chemistry is now intimately involved with the rate-determining step in turnover. Second, 1-deazaFAD-enzyme shows a deuterium-isotope effect on V_{max} greater than 8, slightly higher than that of FAD-enzyme. Thus, 1-deazaFAD reduction is fully rate determining in oxidation of [1-^{2}H]glucose, and at least partially so in oxidation of [^{1}H]glucose. This suggests that the low V_{max} of 1-deazaFAD-enzyme is a reflection of slower reduction of 1-deazaFAD than FAD in catalysis, as expected from their redox potential difference. Third, the comparable K_m values for glucose with FAD- and 1-deazaFAD-enzyme suggest that the physical steps of substrate binding and release are not drastically altered by coenzyme substitution. Bright and Gibson (1967) have shown that K_m approaches K_s for those substrates with low V_{max} values, so that K_m is reasonably independent of V_{max}. Fourth, the K_{m,O_2} values give apparent bimolecular rate constants for enzyme oxidation ($\equiv V_{max}/K_{m,O_2}$) that are approximately the same for FAD- and 1-deazaFAD-enzymes, and are not dependent on glucose deuteration. This is reassuring, since we expect that dihydro-1-deazaFAD should be oxidized at at least the rate of dihydroFAD (vide supra; Spencer et al., 1977).

Conclusions

These data accumulated with 1-deazariboflavin and 1-deazaFAD with the three enzymes NAD(P)H:flavin oxidoreductase, D-amino acid oxidase, and glucose oxidase prove the competence of the 1-deazaflavins in complete oxidative turnover. Thus, a nitrogen atom at position 1 of the isoalloxazine nucleus is *not* essential either for biological reduction or oxidation. At this point we may consider the complementary uses of the two carba-deaza flavin isosteres, 5-deazaflavins, and 1-deazaflavins. These two classes of flavin analogues, with carbon substitution at the two termini of the 1,5 redox center, provide surprisingly different types of mechanistically useful information about the chemistry and enzymology of flavin redox catalysis.

We and others (Fisher et al., 1976; Hersh and Jorns, 1976) have used 5-deazaflavins in several enzyme systems, with the principal conclusion that, in every case, hydrogen from the oxidized substrate is transferred directly and completely to position 5 of the 5-deazaflavin coenzyme. However, since dihydro-5-deazaflavins are not readily oxidized by molecular oxygen, 5-deazaflavin-substituted flavoenzyme oxidases are competent in their reductive half-reactions but not in the oxidative half-reactions.

In contrast, the 1-deazaflavins are fully competent in both one- and two-electron processes; indeed, the only immediately notable differences between the 1-deazaflavins and the natural flavins are their different redox potentials and the striking purple color and total lack of fluorescence of the 1-deazafla-

vins. Many of our conclusions to date are based on correlations between enzymatic observables (V_{max} and K_m values, isotope effects, alternative pathway partitionings) and the redox potentials of the 1-deazaflavins (−280 mV) vs. the natural flavins (−208 mV). With the *B. harveyi* oxidoreductase and glucose oxidase, V_{max} values for the 1-deazaflavin enzymes are 2.5 and 10% those with the natural flavins, respectively; flavin (or 1-deazaflavin) reduction is at least partially rate determining for both enzymes. D-Amino acid oxidase exhibits the same V_{max} values with either FAD or 1-deazaFAD as coenzyme; we have several indications that a physical step, product release, is rate determining in catalysis with 1-deazaFAD as well as FAD-D-amino acid oxidase. Thus, any kinetic consequences of the low redox potential of 1-deazaFAD are masked by the slow, coenzyme-independent step of product release.

The three enzymes examined in this paper are flavoprotein oxidases. There are two other categories of flavoenzymes which may well merit examination on 1-deazaflavin substitution. One such is that of flavoprotein dehydrogenases; of particular mechanistic interest here will be the discrete multienzyme complexes involved in camphor or steroid hydroxylation, where a flavoprotein accepts two electrons from NADH and passes them, one at a time, to a non-heme iron-sulfur protein (e.g., putidaredoxin, adrenodoxin) which in turn delivers electrons to the actual hydroxylating component, the cytochrome P_{450} enzyme. A controlled change in flavin redox potential of 70 mV (by preparation, e.g., of 1-deazaFAD-adrenodoxin reductase) may alter the rates of electron transport and help dissect the complex redox mechanism.

The second category is flavoprotein monooxygenases (Massey and Hemmerich, 1975). The change in redox potential may alter the fate of the dioxygen molecule which is normally activated and split with insertion of one oxygen atom into substrate. In fact, in preliminary studies with 1-deaza-FAD-orcinol hydroxylase (Cheung, Cheung, and Walsh, unpublished observations), we have observed an *uncoupling* of NADH oxidation from orcinol hydroxylation with O_2 being exclusively reduced to H_2O_2 rather than split for substrate hydroxylation. Orcinol remains essential for 1-deazaFAD-enzyme catalysis, but as effector and not as a substrate.

Acknowledgments

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