Stereospecificity in the Oxidation of NADH by Flavopapain

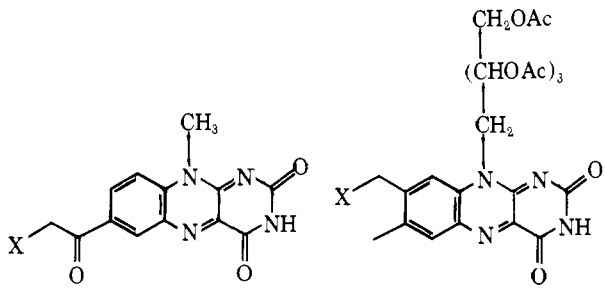
Howard L. Levine and E. T. Kaiser*

Contribution from the Department of Chemistry, University of Chicago, Chicago, Illinois 60637. *Received June 18, 1979*

Abstract: The stereochemistry of the hydride transfer reaction between dihydronicotinamides and flavopapain **3** was examined by studying the oxidation of [4A-^{2}H]NADH, [4B-^{2}H]NADH, NADH, and [4AB-$^2H_2$]NADH. The oxidation of [4A-^{2}H]NADH by **3** results in the transfer of 68% deuterium from the 4A position of the dihydronicotinamide ring, while oxidation of [4B-^{2}H]NADH results in transfer of 88% hydrogen from the 4A position. These results coupled with kinetic measurements lead to a calculated ratio of 4A to 4B hydrogen transfer in NADH of 7. This preference for transfer from the 4A position can be understood in terms of the postulated structure of the NADH-flavopapain complex.

Introduction

In the course of our research on the "chemical mutation" of enzyme active sites leading to the formation of semisynthetic enzymes, we have described the preparation of flavopapains **3** and **6** by the alkylation of the essential thiol group of Cys-25 of papain by 7α-bromoacetyl-10-methylisoalloxazine (**2**) and 8α-bromo-2′,3′,4′,5′-tetra-*O*-acetylriboflavin (**5**), respectively.[1-3] Flavopapain **3** is an effective oxidoreductase showing normal flavoenzyme behavior in its reactions with dihydronicotinamides, exhibiting saturation kinetics at low substrate concentrations and significant rate accelerations when compared with the corresponding reactions of the model compound 7-acetyl-10-methylisoalloxazine **1** with the dihydronicotinamides.[1,3] In the present article we describe our first study of the stereochemical behavior of flavopapain **3**. We have examined the stereochemistry of the hydride transfer reaction between NADH and this flavoenzyme.



1, X = H
2, X = Br
3, X = S—papain

4, X = H
5, X = Br
6, X = S—papain

Experimental Section

General. Proton magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker HS-270 spectrometer equipped with a Nicolet NIC-80 Fourier transform system. All spectrophotometric determinations were performed on a Beckman Acta MVI spectrophotometer equipped with a thermostatic bath that held the temperature in the cell compartment to 25.0 ± 0.3 °C.

Materials. Papain used throughout this work was obtained from Sigma as a suspension of twice recrystallized enzyme in 0.05 M acetate, pH 5.0. The following materials were purchased from the companies indicated: NAD$^+$, PL Biochemicals; D,L-lipoamide and lipoamide dehydrogenase, Sigma; yeast alcohol dehydrogenase, Calbiochem; dithiothreitol (DTT), [U-^{2}H]ammonium hydroxide (30% solution in D_2O) and deuterium oxide (99.8 and 100 atom % D), Aldrich; tris(hydroxymethyl)aminomethane (Tris) from Schwarz/Mann; and DEAE cellulose (DE 52), Whatman. Buffers were prepared with doubly distilled deionized water (Continental Demineralizer); all other solvents and reagents were of the highest purity available and were used without further purification.

7-Acetyl-10-methylisoalloxazine 1 and 7α-Bromoacetyl-10-methylisoalloxazine 2. These compounds were prepared from *p*-chloroacetophenone as described previously.[3]

Preparation of Flavopapain 3. Commercially available papain was purified as its mercury derivative by the method of Sluyterman and Widjenes[4] with the exception that Me_2SO and 1-butanol were omitted from the elution buffers. The purified enzyme was then activated and modified as described previously.[3] The concentrations of solutions of flavopapain **3** were determined by using ϵ_{427} 10 900 M^{-1} cm^{-1}.[3]

Deuterated NADH Derivatives.[5] [4B-^{2}H]NADH was prepared by reducing 218.9 mg (300 μmol) of NAD$^+$ with 3.4 mg (15 μmol) of D,L-lipoamide and 40 units of lipoamide dehydrogenase (a B stereospecific enzyme) in the presence of 96.0 mg (600 μmol) of dithiothreitol (DTT) and 25 mL of D_2O (99.8 atom % D). The pD of the reaction solution was adjusted to 8.5 and maintained between 8.4 and 8.7 by addition of [U-^{2}H]ammonium hydroxide. After 45 min the solution was diluted with 15 mL of D_2O and applied to a DEAE-cellulose column (1.8 × 17 cm, carbonate form). Elution of the product from the column was then performed with a 1-L gradient of 0-0.5 M $(NH_4)_2CO_3$. The [4B-^{2}H]NADH was found to elute at approximately 0.15 M $(NH_4)_2CO_3$, and the combined fractions containing [4B-^{2}H]NADH yielded 201.7 mg (275 μmol, 92% yield) which contained 97 ± 3% deuterium in the 4B position as judged by NMR.

[4-^{2}H]NAD$^+$ was prepared by oxidizing the [4B-^{2}H]NADH prepared, as described above, with yeast alcohol dehydrogenase (an A stereospecific enzyme) in the presence of excess acetaldehyde (0.5 M) in 25 mL of 50 mM $(NH_4)_2CO_3$. When the reaction was complete as judged by the decrease in absorbance at 340 nm (usually less than 20 min), the reaction mixture was filtered through a Millipore filter (0.45 μm) and then lyophilized. It was not necessary to purify the [4-^{2}H]NAD$^+$ thus isolated further since no impurities were present and the material contained 97 ± 3% deuterium in the 4 position as judged by NMR.

[4A-^{2}H]NADH was prepared by reducing [4-^{2}H]NAD$^+$ with lipoamide dehydrogenase as described above for the preparation of [4B-^{2}H]NADH except that the reaction was carried out in H_2O. The purified [4A-^{2}H]NADH contained 97 ± 3% deuterium in the 4A position as judged by NMR.

[4AB-$^2H_2$]NADH was prepared by reducing [4-^{2}H]NAD$^+$ with lipoamide dehydrogenase as described for the preparation of [4B-^{2}H]NADH. The purified [4AB-$^2H_2$]NADH contained 96 ± 3% deuterium in both the 4A and 4B positions as judged by NMR.

Determination of the Extent of Hydrogen Transfer in the Oxidation of NADH, [4A-^{2}H]NADH, and [4B-^{2}H]NADH by Flavopapain 3 and 7-Acetyl-10-methylisoalloxazine 1. For the oxidation of NADH by flavopapain **3**, a mixture of 5.6 μM **3** and 0.5 mM NADH in 50 mL of 10 mM $(NH_4)_2CO_3$ in the vicinity of pH 8.5 was allowed to stand at room temperature for 20 h. Subsequently, the solution was filtered through a 0.45-μm Millipore filter and lyophilized. The oxidation of NADH by the flavin **1** was carried out in an analogous manner except that 30 μM flavin was used instead of the enzyme. The extent of hydrogen vs. deuterium transfer in all cases was determined by NMR analysis of the lyophilized residue.

In order to remove excess water and exchangeable protons from the samples prior to NMR analysis, the residues were redissolved in 10 mL of D_2O (99.8 atom % D) and lyophilized a second time. For NMR analysis the residues were dissolved in 0.5 mL of D_2O (100 atom %

0002-7863/80/1502-0343$01.00/0

Table I. Product Ratios for the Oxidation of Deuterated NADH Derivatives by Flavopapain **3** and by Flavin **1** at 25 °C

flavin	NADH derivative	$(4\,^2H)NAD^+/(4\,^1H)$-NAD^+
1	[4A-^{2}H]NADH	2.04
	[4B-^{2}H]NADH	2.86
3	[4A-^{2}H]NADH	0.47
	[4B-^{2}H]NADH	7.33

Table II. Second-Order Rate Constants for the Oxidation of Deuterated NADH Derivatives by Flavin **1** at 25 °C

NADH derivative	k, $M^{-1}\,s^{-1}$
NADH	12.9
[4A-^{2}H]NADH	4.58
[4B-^{2}H]NADH	4.52
[4AB-$^2H_2$]NADH	1.53

D). Fifty to 100 transients were acquired at a sweep width of 3000 Hz with a recycle time of 0.5 s. The recorded free induction decays were processed with an exponential multiplier of 0.3 Hz. Chemical shifts were determined by using the residual protons of HOD present in the sample.

The extent of hydrogen vs. deuterium transfer can be determined from the ratio of the pyridinium C(6) protons (9.11 ppm) to the pyridinium C(4) protons (8.95 ppm). In order to determine this ratio accurately, the region of the NMR spectrum containing the peaks for these protons (7.5–9.5 ppm) was expanded and integrated three times. These three integrals were then averaged (variation ± 2%) to give the recorded integral for a given experiment.

Kinetics. The rate of NADH oxidation was measured in all cases by observing the decrease in absorbance at 340 nm with time. In a typical experiment, 2.0 mL of a 0.1 M Tris-HCl, pH 7.5 solution containing either flavopapain **3** or flavin **1** was pipetted into a 2.5-mL capacity stoppered cuvette with a 1-cm path length. After equilibration at 25.0 °C, the reaction was initiated by adding an aliquot of a solution of NADH in 0.1 M Tris-HCl, pH 7.5.

In the case of flavin **1** catalyzed oxidation, pseudo-first-order rate constants, k_{obsd}, were obtained from the entire time course of the reaction. These were converted to second-order rate constants, k, by the use of eq 1:[3]

$$k = k_{obsd}/(\mathbf{1}) \tag{1}$$

For flavopapain **3** catalyzed oxidation, initial rates corresponding to less than 5% total reaction time were utilized in constructing Lineweaver-Burk plots.

Results

Isotope Partitioning Ratios in the Oxidation of [4A-^{2}H]NADH and [4B-^{2}H]NADH by 7-Acetyl-10-methylisoalloxazine 1 and by Flavopapain 3. The stereochemistry of the hydride transfer reaction between NADH and flavopapain **3** was studied utilizing the deuterated NADH derivatives [4A-^{2}H]NADH and [4B-^{2}H]NADH. In order to examine this reaction, we employed the NMR method of Arnold et al.[4] in which the stereospecifically deuterated NADH derivatives are oxidized to yield either [4-^{2}H]NAD^+ or [4-^{1}H]NAD^+. The extent of retention of a proton at the 4-position of the pyridine ring can be quantitatively determined by comparing the integration of its resonance peak at 8.95 ppm with that for the proton at the 6-position of the pyridine ring (9.11 ppm). The product ratios [4-^{2}H]NAD^+/[4-^{1}H]NAD^+ determined by this method for the oxidation of [4A-^{2}H]NADH and [4B-^{2}H]NADH by flavopapain **3** and by flavin **1** in 10 mM $(NH_4)_2CO_3$ at room temperature are listed in Table I.

Isotope Effects on the Rate of Oxidation of NADH by Flavin 1 and Flavopapain 3. Under dilute conditions, the rate of oxidation of NADH, [4A-^{2}H]NADH, [4B-^{2}H]NADH, and [4AB-$^2H_2$]NADH by the flavin **1** obeys simple second-order kinetics. The second-order rate constants which we obtained at pH 7.5 in 0.1 M Tris-HCl at 25.0 °C are listed in Table II.

Table III. Rate Parameters for the Oxidation of Deuterated NADH Derivatives by Flavopapain **3** at 25 °C

NADH derivative	k_{cat}/K_m, $M^{-1}\,s^{-1}$
NADH	68.1
[4A-^{2}H]NADH	17.3
[4B-^{2}H]NADH	43.6
[4AB-$^2H_2$]NADH	3.2

Under aerobic conditions, the oxidation of all deuterated NADH derivatives by flavopapain **3** followed saturation kinetics, as also observed in the oxidation of other dihydronicotinamides by this semisynthetic enzyme. The observed kinetics are readily explained by the scheme shown in eq 2 and 3, where E and E′ represent the oxidized and reduced enzyme, respectively, ES represents a Michaelis complex between enzyme and substrate, and K_m is the dissociation constant for this complex. In Table III the values of k_{cat}/K_m which we have obtained for each NADH derivative at pH 7.5 in 0.1 M Tris-HCl, 25.0 °C, are listed.

$$E + NADH \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E' + NAD^+ \tag{2a}$$

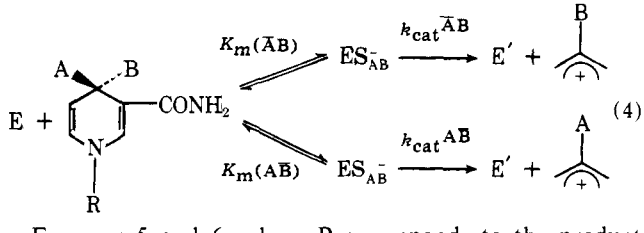
$$E' + O_2 \xrightarrow{k_0} E + H_2O_2 \tag{2b}$$

$$k_0(O_2) \gg k_{cat} \tag{2c}$$

$$v = \frac{k_{cat}(E)_0(NADH)}{K_m + (NADH)} \tag{3}$$

Discussion

From the results listed in Tables I–III, it is quite clear that flavopapain **3** shows significant, but not complete, preference for transfer of the 4A (*pro-R*) hydrogen of NADH to the covalently bound flavin moiety of the enzyme. Assuming that this stereospecificity results from different rates of hydride transfer from the A and B sides of the dihydronicotinamide ring, we can use the scheme of eq 4 to explain our results. In this scheme, A represents the hydrogen (or ^{2}H) on the A side of the dihydronicotinamide ring and B represents the group on the B side. The bar written above A or B denotes the hydrogen (or ^{2}H) being transferred. The air oxidation of the reduced enzyme E′ back to the oxidized form E is postulated to occur rapidly relative to the hydride transfer process under the conditions of our experiments (see eq 2c).



(4)

From eq 5 and 6, where P corresponds to the product formed, eq 7 for the ratio of B-containing product to A-containing product is readily obtained. Combining eq 7 with eq 8 leads to eq 9 and 10.

$$dP_B/dt = k_{cat}^{\bar{A}B}[ES_{\bar{A}B}] \tag{5}$$

$$dP_A/dt = k_{cat}^{A\bar{B}}[ES_{A\bar{B}}] \tag{6}$$

$$P_B/P_A = (k_{cat}/K_m)_{\bar{A}B}/(k_{cat}/K_m)_{A\bar{B}} \tag{7}$$

$$(k_{cat}/K_m)_{AB} = (k_{cat}/K_m)_{\bar{A}B} + (k_{cat}/K_m)_{A\bar{B}} \tag{8}$$

$$(k_{cat}/K_m)_{\overline{A}B} = \frac{(k_{cat}/K_m)_{AB}[(P_B/P_A)]}{[(P_B/P_A) + 1]} \quad (9)$$

$$(k_{cat}/K_m)_{A\overline{B}} = \frac{(k_{cat}/K_m)_{AB}}{[(P_B/P_A) + 1]} \quad (10)$$

In principle the ratio for the rate of hydrogen transfer from the A side to that from the B side could be obtained from a simultaneous solution of eq 8–10 and the data of Tables I and III. However, we were unable to obtain a meaningful solution in this way. One reason for this may be that the experimental error in the product ratios, as determined by NMR, is significant (>5%). Another possible problem is that nonstereospecific exchange of the C(4) hydrogens of the product NAD^+ and the reactant NADH is possible. This exchange has been found to proceed to the extent of 39% in 8 h when 30 mM [4-^{2}H]NAD^+ and 30 mM NADH were mixed at pH 8, 30 °C.[6] When the concentrations were lowered to 0.3 mM, the chemical exchange was slowed to only 7% in the same period. In the work of Arnold et al.,[5] coenzyme concentrations were kept below 0.5 mM and, since the reactions studied were complete in less than 30 min, nonstereospecific exchange was not a problem. In our work, reaction times of up to 20 h were necessary to convert NADH to NAD^+ quantitatively so that some exchange is probably occurring. In order to minimize exchange in our experiments, we used NADH concentrations of less than 0.5 mM. If nonstereospecific exchange is occurring, then it will have the effect of reducing the measured product isotope ratio. If this isotope effect is large, then even a small amount of scrambling will cause a large error. Consequently, the product isotope ratios reported in Table I represent minimum values.

Additional complications in analyzing the rate and product formation data may arise from the possible presence of kinetically significant intermediates along the reaction pathway leading from ES to E′ + product (eq 4).[7] Since we have not found it feasible to carry out a complete solution of eq 8–10 using our data, we have instead estimated the ratio of rate constants, R, for hydrogen transfer from the A face vs. the B face, as expressed in eq 11, by the use of eq 12 and 13 which hold if secondary isotope effects are neglected.

$$R = \frac{(k_{cat}/K_m)_{\overline{H}H}}{(k_{cat}/K_m)_{H\overline{H}}} \quad (11)$$

$$(k_{cat}/K_m)_{\overline{H}D} \cong (k_{cat}/K_m)_{\overline{H}H} \quad (12)$$

$$(k_{cat}/K_m)_{D\overline{H}} \cong \frac{(k_{cat}/K_m)_{\overline{H}H}}{R} \quad (13)$$

Equation 14 follows from eq 12 and 13, and calculation of the value of R can be carried out by substituting the appropriate experimental parameters given in Tables I and III into eq 9 and 10. In this way R is calculated to be about 7. In other words, there is an approximately sevenfold preference for transfer to flavopapain **3** of hydrogen from the A face of NADH relative to transfer from the B face.

$$\frac{(k_{cat}/K_m)_{\overline{H}D}}{(k_{cat}/K_m)_{D\overline{H}}} \cong R \quad (14)$$

By examining the three-dimensional model of papain in which the flavin moiety has been covalently attached to Cys-25,[3,8] the preference of flavopapain **3** for the 4A hydrogen of NADH can be understood. Based on our results with other dihydronicotinamides, we postulate that the dihydronicotinamide ring of NADH is positioned such that C(4) of the substrate is near the α carbon of Trp-26.[3,8] Furthermore, the flavin and dihydronicotinamide rings must lie parallel to one another in order for hydride transfer to occur.[9] This parallel alignment can be achieved in one of two manners. In the first possible binding mode, the dihydronicotinamide ring is oriented such that the carboxamide side chain at C(3) is pointing toward the exterior of the protein and the surrounding solvent. Such an orientation would lead to transfer of the 4B hydrogen. Transfer of the 4A hydrogen will occur if the plane of the dihydronicotinamide ring is rotated 180°. In this orientation, the carboxamide side chain is lying in the interior of the protein with both the carbonyl and NH_2 groups near potential hydrogen bond donating and accepting groups on the protein. A slight stabilization of this binding mode by hydrogen bonds would lead to the observed preference for hydrogen transfer from the 4A position.

Acknowledgements. Our work was supported in part by National Science Foundation Grant AER 77-14529 (E.T.K.) and by National Research Service Award HS-5-BM-17151 from the National Institute of General Medical Sciences (H.L.L.). We wish to thank Professor Richard L. Schowen of the University of Kansas for helpful discussions which led to the kinetic analysis presented in this paper.

References and Notes

(1) Levine, H. L.; Nakagawa, Y.; Kaiser, E. T. *Biochem. Biophys. Res. Commun.* **1977,** *76,* 64.
(2) Otsuki, T.; Nakagawa, Y.; Kaiser, E. T. *J. Chem. Soc., Chem. Commun.* **1978,** 457.
(3) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1978,** *100,* 7670.
(4) Sluyterman, L. A. Ae; Widjenes, J. *Biochim. Biophys. Acta* **1970,** *200,* 593.
(5) Arnold, L. J., Jr.; You, K.; Allison, W. S.; Kaplan, N. O. *Biochemistry* **1976,** *15,* 4849.
(6) Ludwig, J.; Levy, A. *Biochemistry* **1964,** *3,* 373.
(7) A number of examples of model system reactions involving hydrogen transfer from dihydronicotinamides to acceptors have been reported where differences in isotope effects measured kinetically and by product analysis have been interpreted to indicate that important intermediates lie along the reaction pathway and that the partitioning of these intermediates, going back to the starting compounds or forward to the products, leads to the observed differences. See for instance: Steffens, J. J.; Chipman, D. M. *J. Am. Chem. Soc.* **1971,** *93,* 6694. Creighton, J. D.; Haydu, J.; Mooser, G.; Sigman, D. S. *Ibid.* **1973,** *95,* 6855.
(8) Levine, H. L. Ph.D. Thesis, University of Chicago, 1978.
(9) Bruice, T. C. *Prog. Bioorg. Chem.* **1976,** *4,* 1.