Deuterium Isotope Effects for the Nonenzymatic and Glutamate Dehydrogenase Catalyzed Reduction of an α-Imino Acid by NADH

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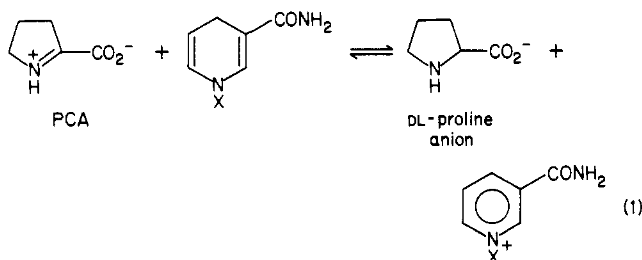
Abstract: The mechanisms of the nonenzymatic and glutamate dehydrogenase catalyzed reduction of an α-imino acid, Δ^1-pyrroline-2-carboxylic acid, by NAD(P)H have been studied by deuterium isotope effects. The partition isotope effects for the nonenzymatic reaction with 4-deuterated 1,4-dihydronicotinamides are about the same as the corresponding observed kinetic isotope effects with 4,4-dideuterio-1,4-dihydronicotinamides, suggesting that the hydrogen-transfer step is solely rate limiting. This reaction is characterized by an intrinsic primary kinetic isotope effect of 1.3 and a very product-like transition state. The enzymatic reaction has been studied by determining the second-order rate constants for the reduction of the imino acid by the enzyme-NADH complex with 4,4-dideuterio and stereospecifically labeled 4-deuterio NADH. The primary isotope effect when the in-place hydrogen is protium is 3.80, and the secondary isotope effect when the in-flight hydrogen is protium is 1.21. Deuteration at one site lowers the isotope effects at the other by 13%. The following conclusions emerge for the reduction of the imino acid by the enzyme-NADH complex: (1) the hydrogen-transfer step is at least rate contributing, (2) the transition state for this reaction is more symmetric than that of the nonenzymatic reaction, (3) both the C-4 hydrogens of NADH participate in the reaction coordinate motion, and (4) there is some nuclear tunneling in the reaction coordinate. The kinetic isotope effect for the oxidation of proline and proline-*2-d* by enzyme-NADP$^+$ is 4.1.

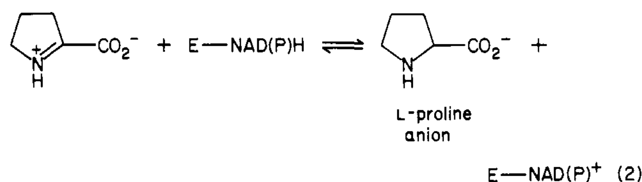
Although the reductions of specific oxidants by dihydronicotinamides are described as hydride-transfer reactions, it is not always clear whether the hydride-transfer process proceeds through a single kinetic event or by a multistep mechanism.[1] In addition to this ambiguity, nonconserved bond order for the in-flight hydrogen at the transition state[2] and the presence of covalent reactive intermediates[3] are indicated for some hydride-transfer reactions. It has become apparent that deuterium isotope effect studies are very useful in addressing these mechanistic details and in characterizing the transition-state structure.[4]

In connection with our studies on the catalysis of the reductive amination of α-ketoglutarate by glutamate dehydrogenase, we have previously reported the reduction of Δ^1-pyrroline-2-carboxylic acid (PCA) by 1,4-dihydronicotinamides (eq 1).[5]



While the reductin of the cyclic iminium ion by NADPH to give proline anion and NADP$^+$ is found to have a $\Delta G°$ of -3.3 kcal/mol (at 25 °C),[6] a unit Brønsted coefficient results when the rates for the reduction of the imino acid by N - 1 substituted 1,4-dihydronicotinamides are correlated with the latter's redox potentials.[5] The same reaction with 4,4-dideuterated 1,4-dihydronicotinamides gives modest observed kinetic isotope effects[5] of about 1.5.

The reduction of PCA is also catalyzed by glutamate dehydrogenase[7] when the reductant is NAD(P)H, eq 2, and the reverse reaction rate now becomes measurable.[6] The enzymatic



reaction, shown in eq 2, involves the protonated imine and the conjugate base of L-proline as the active substrates.[6] We have been unable to detect any enzyme-coenzyme-substrate ternary complex in either direction under the experimental conditions.

We now report the partition isotope effects for the nonenzymatic reaction with 1,4-dihydronicotinamides monodeuterated at C-4 and compare them with the kinetic isotope effects obtained with 4,4-dideuterated dihydronicotinamides. Although the separation of observed kinetic isotope effects for the nonenzymatic reaction into primary and secondary is difficult,[5,8] these isotope effects are easily determined for the enzymatic reaction since the hydride transfer is absolutely stereospecific only for the enzymatic reaction. We also report these kinetic isotope effects and apply the data to probe the transition-state structure of the enzymatic reaction. The isotope effects are interpreted in terms of current theories pertaining to quantum mechanical tunneling and coupling between the in-flight and in-place hydrogens of NADH along the reaction coordinate.

Experimental Section

Materials. Ethyl alcohol-d_6 (≥99 atom % D) from Merck Sharp and Dohme and NADH, NADPH, reduced 3-acetylpyridine adenine dinucleotide, NADP$^+$, and NAD$^+$ from Sigma Chemicals were used without further purification. Bovine liver glutamate dehydrogenases was obtained as an ammonium sulfate suspension from Boehringer Mannheim and treated with Norit A to remove nucleotides.[9]

1-Methyl-, 1-benzyl-, and 1-(carbamoylmethyl)-1,4-dihydronicotinamides monodeuterated at C-4 were synthesized (> 0.96 d at C-4 by NMR) by the reduction of the corresponding pyridinium salt with sodium dithionite in D_2O (≥99.8 atom % D) with use of the general method of

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Caughey and Schellenberg.[10] The 4-deuterated derivatives of NADH were prepared as barium salts.[11] Prior to use, they were treated with sodium sulfate solution (50 mM) in 0.01 M phosphate buffer (pH 7.6) and the precipitated barium sulfate was removed by centrifugation. The concentrations of glutamate dehydrogenase and the coenzymes were determined spectrophotometrically.[12]

α-Keto-δ-aminovaleric acid was synthesized as the hydrochloride salt.[13] This compound spontaneously cyclizes[14] to Δ^1-pyrroline-2-carboxylic acid (PCA) upon dissolution in water at pH >2. L-Proline from Sigma Chemicals and DL-proline-*2-d* (99.2 atom % D) from Merck Sharp and Dohme were purified by repeated crystallization of the amino acid from aqueous ethanol.

Partition Isotope Effect. The amount of D and H at C-4 in the 1-substituted 3-carbamoylpyridinium ion, derived from the oxidation of the corresponding 1,4-dihydroicotinamide-*4-d* by PCA, was determined by NMR. In a typical experiment, PCA (1.2 mmol) was dissolved in 4 mL of D_2O, and the pD of the solution was adjusted to 6.2 with DCl/D_2O. 1-Methyl-1,4-dihydronicotinamide-*4-d* (0.5 mmol) was added and the solution kept stirred in nitrogen atmosphere maintaining the pD at 6.2 to 6.5. After an hour, the NMR spectrum of the solution was recorded. The partition isotope effect was calculated by eq 3 where Q represents the intensity of C-5 proton (multiplet centered at δ 8.2) and S the intensity of C-6 and C-4 protons together (multiplet centered at δ 9.0) in the product pyridinium ion. In the case of 1-benzyl-1,4-di-

$$\text{partition isotope effect} = \frac{2Q - S}{S - Q} \tag{3}$$

hydronicotinamide-*4-d*, the dissolution of the reductant took about an hour, during which period the pD was maintained at 6.2 to 6.5 with DCl/D_2O. The reaction times for the 1-benzyl and 1-carbamoylmethyl substituted dihydropyridines were 2 and 7 h, respectively.

Isotope Effects for the Enzymatic Reduction of PCA. The reaction rates were measured in 0.1 M Tris, 0.01 M phosphate buffer spectrophotometrically as described previously.[6] Stock solutions of PCA in Tris–phosphate were prepared and the pH adjusted close to the desired value with concentrated KOH. Stock solutions of NADH and its isotopic variants in 0.01 M phosphate–0.05 M sodium sulfate (pH ~7.6) were also prepared and kept on ice. A portion of the PCA solution was treated with glutamate dehydrogenase in 0.1 M phosphate (pH 7.60), and the pH of the resulting solution at 25 °C was recorded. Two milliliters of the PCA–enzyme stock solution was transferred to the cuvette and allowed to thermally equilibrate in the cell compartment. The reaction was initiated by adding the conenzyme solution (10 to 20 μL) and the initial rate determined as previously described.[6] The rate constants with the deuterated analogues of NADH were measured similarly by using the same PCA–enzyme stock solution. The pH of the solutions before and after rate measurements remained the same. Since NADH synthesized in parallel with [4,4-^{2}H]NADH had the same reactivity as the commercial sample, the latter was used in the measurement of isotope effects.

The observed initial rates in the enzymatic reaction must be corrected for the contribution due to the nonenzymatic reaction rates. These blank rates were determined with the second portion of the PCA solution which was treated with 0.1 M phosphate buffer (at pH 7.60), the volume of the buffer added being equal to that of the enzyme. The contribution of the blank reaction rate to the observed rate is less than 2%. The rate constants, which are quite sensitive to the concentration of the enzyme and pH of the medium,[6] are subject to errors of ±5%. However, the isotope effects were measured under identical conditions by adding the isotopic variants of NADH to the same PCA–enzyme stock solution. The kinetic isotope effects represent the average values from five determinations; the standard deviations are about 3%. These errors are much smaller than the 7% which would have resulted from measuring the individual rates in an uncorrelated fashion.

Isotopic Purity. The magnitudes of isotope effects are affected by the isotopic purity of the reductants. The isotopic purity of [4,A-^{2}H]NADH, which was enzymatically synthesized from 99%+ deuterated ethanol, was confirmed by nuclear magnetic resonance. A comparison of the intensities of the C-4 (δ 6.9) and C-2 (δ 2.7) protons of [4,4-^{2}H]NADH revealed that this coenzyme contains at least 96% deuterium at the 4B position. A protium contamination of 4% at the 4B position in the 4B-deuterated NADH would change[15] an observed isotope effect of 3.80 to a corrected value of 4.30. Since the conclusions presented are not affected by the corrections we have ignored them in this report.

Isotope Effect for the Enzymatic Oxidation of L-Proline. The rate for the enzymatic oxidation of proline by $NADP^+$ was obtained by following the appearance of NADPH at 340 nm.[6] The isotope effect was determined from rate measurements with L-proline (0.5 M) and DL-proline-*2-d* (1.0 M) under identical conditions (0.1 M Tris, 0.01 M phosphate, [E] = 2 mg/mL, [$NADP^+$] = 2 mM, and pH 9.45). Since the pH measurements of the proline–enzyme solution are subject to an error of ±0.05, these isotope effects have an estimated error of ±10%. We have previously reported that D-proline is neither a substrate nor an inhibitor for glutamate dehydrogenase.[6]

Table I. Partition Isotope Effects and k^{HH}/k^{DD} Ratios[a] for the Nonenzymatic Reduction of PCA by $N-1$ Substituted 1,4-Dihydronicotinamides

compd no.	substituent at $N-1$	k^{HH}/k^{DD}	partition isotope effect
1	CH_3[b]		1.1 ± 0.2
2	CH_2–C_6H_5	1.57 ± 0.08	1.2 ± 0.3
3	CH_2–$CONH_2$	1.54 ± 0.08	1.1 ± 0.3
4	NADH[c]	1.53 ± 0.08	

[a] Taken from ref 5; k^{HH} and k^{DD} represent the second-order rate constants for the reduction of PCA by dihydronicotinamide and by 4,4-dideuterated dihydronicotinamide, respectively. [b] k^{HH}/k^{HD} = 1.09 (ref 5). [c] Identical rate constants were observed for [4A-^{2}H]NADH and [4B-^{2}H]NADH.

Table II. Rate Constants and Isotope Effects for the Enzyme-Catalyzed Reduction of PCA[b]

coenzyme, R	$10^2 k_E^R$,[a] M^{-1} s^{-1}
[4,4-^{1}H]NADH	9.85
[4,A-^{2}H]NADH	8.14
[4,B-^{2}H]NADH	2.59
[4,4-^{2}H]NADH	2.45
$(PIE)_H$ = 3.80; $(PIE)_D$ = 3.32	
$(SIE)_H$ = 1.21; $(SIE)_D$ = 1.06	

[a] k_E^R represents the rate of the reaction for the reduction of 1 M total PCA by 1 M E–R complex. [b] [PCA] = 51.2 mM; [E] = 2.3 mg/mL; pH 8.03; [Tris] = 0.1 M; [PO_4^{3-}] = 0.01 M; [reduced coenzyme] = 200 μM.

Results

Isotope Effects in the Nonenzymatic Reduction of PCA. The partition isotope effects for the PCA reaction (as measured by the relative amounts of protium and deuterium at C-4 in the product pyridinium ion) were obtained with three dihydronicotinamides, each of which was monodeuterated at the 4-position. These isotope effects along with the corresponding k^{HH}/k^{DD} ratios[5] are collected in Table I. It is apparent that there is no significant difference between the magnitudes of k^{HH}/k^{DD} ratios and the partition isotope effects.

Isotope Effects for the Enzymatic Reduction of PCA. The rate constants and the isotope effects observed with NADH and its 4-deuterated analogues are listed in Table II. The difference in the reactivity of the A and B side hydrogens of NADH arises from the stereospecific transfer of hydrogen from the B side of the coenzyme in the enzymatic reaction. Consequently, the kinetic isotope effects are obtained as simple ratios of rate constants as shown in eq 4–7 where k_E^R is the second-order rate constant for the reduction of PCA by the E–R complex. The primary isotope

$$(PIE)_H = k_E^{[4,4\text{-}^1H]NADH} / k_E^{[4,B\text{-}^2H]NADH} \tag{4}$$

$$(PIE)_D = k_E^{[4,A\text{-}^2H]NADH} / k_E^{[4,4\text{-}^2H]NADH} \tag{5}$$

$$(SIE)_H = k_E^{[4,4\text{-}^1H]NADH} / k_E^{[4,A\text{-}^2H]NADH} \tag{6}$$

$$(SIE)_D = k_E^{[4,B\text{-}^2H]NADH} / k_E^{[4,4\text{-}^2H]NADH} \tag{7}$$

effects when the in-place α-H atom of NADH (A side) is protium and deuterium are represented by $(PIE)_H$ and $(PIE)_D$, respectively.

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Table III. Dependence of Kinetic Deuterium Isotope Effects on the Concentrations of PCA and Coenzyme for the Enzymatic Reduction of PCA[b]

[coenzyme],[a] μM	[PCA], mM	$(PIE)_D$	$(SIE)_H$
195	51.2	3.38	1.19
98	51.2	3.14	
47	51.2	3.25	1.24
195	26.0	3.19	
195	80.4	3.32	1.18

[a] K_m for NADH = 17 ± 4 μM. [b] T = 25 °C; [Tris] = 0.1 M; $[PO_4^{3-}]$ = 0.01 M; [E] = 2.4 mg/mL; pH 7.13.

Similarly, $(SIE)_H$ and $(SIE)_D$ are the secondary isotope effects when the in-flight α-hydrogen atom (B side) is protium and deuterium, respectively.

Table III gives measurements of PIE's and SIE's over a range of coenzyme and PCA concentrations. The data show that these isotope effects are essentially independent of substrate concentration.

The isotope effects, measured as a function of temperature over the range of 7 to 34 °C, do not show any significant changes with temperature beyond those calculated[16] from the exponential temperature dependence.

The isotope effects were determined over the pH range 6.2–9.0. The $(PIE)_D$'s and $(SIE)_H$'s for the enzymatic PCA reaction vary with no apparent trend from 3.21 to 3.47 and from 1,18 to 1.24, respectively.

In contrast to NADPH, NADH binds to a second nonactive site on glutamate dehydrogenase; the rates for the enzyme-catalyzed reductive amination of α-ketoglutarate are altered as a result of such binding.[17] Since we use NADH and its deuterated analogues in our studies, it is necessary to determine if such effects occur in the enzymatic PCA reaction. To this end, we compared the kinetic parameters for these coenzymes in the enzyme-catalyzed reduction of PCA. The K_m values for NADPH and NADH at pH 7.10 were determined to be 13 ± 3 and 17 ± 4 μM respectively; the enzyme–NADPH complex at the same pH was found to be a more effective reductant than the enzyme–NADH complex by just 15 ± 3%. The correspondence of these parameters suggests that the redox step itself is not affected by the absence of the phosphate group in the NADH molecule at position 2′ of the ribose in the adenylic acid moiety and that the study of isotope effects with NADH and its deuterated analogues is not clouded by the binding of this coenzyme to the nonactive site.

Isotope Effect in the Enzymatic Oxidation of Proline. We determined the primary isotope effect for the oxidation of L-proline and DL-proline-*2-d* by $NADP^+$ at pH 9.42. The PIE for this reaction was found to be 4.1 ± 0.4. The D isomer has no effect on the L-proline reaction rates.[6]

Discussion

Nonenzymatic Reaction. In a previous study,[5] we noted that the k^{HH}/k^{DD} ratio for the nonenzymatic reduction of PCA is about 1.5 (Table I) which was interpreted as a PIE, the SIE having been assumed to be unity. In the present study, we examine the reaction for any kinetic complexity that might be responsible for the low k^{HH}/k^{DD} ratios.

Low observed isotope effects (k^{HH}/k^{DD} ratios) in hydride-transfer reactions might arise due to any one of the following factors: (1) the reaction has a substantial inverse secondary isotope effect;[18] (2) a kinetically significant intermediate intervenes prior to the hydrogen-transfer step;[19] (3) an isotopically insensitive nonredox reaction of the same kinetic order as that of the redox reaction is competing with the reduction process;[20] or (4) the redox step itself has a low intrinsic primary isotope effect. Factor 1 will result in partition isotope effects that are larger than the k^{HH}/k^{DD} ratios. Factors 2 and 3 should also produce similar disparities[3,21] which are likely to change as the substituent in the dihydropyridine is varied. Factor 4 will produce agreement between k^{HH}/k^{DD} ratios and the partition isotope effects, when SIE is unity. We observe that the kinetic and partition isotope effects for the PCA reaction (Table I) do not differ significantly with various dihydronicotinamides whose reactivities span a range of 39-fold. We conclue that the k^{HH}/k^{DD} ratios represent a combination of intrinsic primary and secondary kinetic isotope effects for the PCA reaction.

The hybridization of C-4 of the dihydropyridine changes from sp^3 to sp^2 for a rate-limiting hydride transfer from the reductant; the SIE must then be greater than unity. Since k^{HH}/k^{DD} = (PIE)(SIE) and the partition isotope effect = PIE/SIE, the magnitude of k^{HH}/k^{DD} is expected to be larger than that of the latter. The data in Table I are indeed consistent with this expectation.

The SIE for the reaction can be calculated from the corresponding secondary equilibrium isotope effect, $(SIE)_{eq}$, in eq 8 where DHP and Py^+ represent the dihydropyridine and pyridinium ion, respectively. The Leffler relation predicts that SIE will vary

$$(SIE)_{eq} = \frac{[Py^+][DHP\text{-}4\text{-}d]}{[Py^+\text{-}4\text{-}d][DHP]} \quad (8)$$

from unity to $(SIE)_{eq}$ as shown in eq 9 where β represents the fractional displacement of the transition state along the reaction coordinate.[22] Since β^5 and $(SIE)_{eq}$[23] are 1.0 and 1.127, respec-

$$SIE = (SIE)_{eq}^{\beta} \quad (9)$$

tively, a value of 1.127 applies for the SIE in the nonenzymatic PCA reaction. It follows that the intrinsic primary kinetic isotope effect corresponding to the k^{HH}/k^{DD} ratio of 1.5 for the reaction is 1.3.

The low PIE of 1.3 must be interpreted with reference to $(PIE)_{eq}$, the appropriate equilibrium isotope effect (eq 10), since PIE ≥ $(PIE)_{eq}$ for a very product-like transition state.[16] Here,

$$(PIE)_{eq} = \frac{[Pr^-][DHP\text{-}4\text{-}d]}{[Pr^-\text{-}2\text{-}d][DHP]} = \frac{\phi_{DHP}}{\phi_{Pr^-}} \quad (10)$$

ϕ_{DHP} and ϕ_{Pr^-} are the fractionation factors relative to acetylene for hydrogen on C-4 of DHP and C-2 of Pr^-, the proline anion, respectively. The value of ϕ_{DHP} is calculated[24] to be 1.53, while eq 11 shows the calculation of ϕ_{Pr^-}. We obtain $\phi_{CH_3CHDNH_2}$ relative

$$\phi_{Pr^-} = \phi_{CH_3CHDNH_2}(\phi_{CH_2DNO_2}/\phi_{CH_3D}) \quad (11)$$

to acetylene from its fractionation factor relative to ethane[25] and that of ethane relative to acetylene.[26] The fractionation factors of nitromethane and methane have been reported by Hartshorn and Shiner.[26] We have assumed that the nitro substituent is a model for the carboxylate group of Pr^- and have calculated a ϕ_{Pr^-} of 1.84 and $(PIE)_{eq}$ of 0.83. The latter value is in excellent agreement with the experimental value of 0.82 calculated from the fractionation factors of NADH[27] and proline[28] of 0.98 and

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1.20 (relative to water), respectively. The calculated PIE of 1.3 is indeed significantly larger than 0.83 and supports a product-like transition state for the nonenzymatic reduction of PCA.

Mechanism of the Nonenzymatic Reaction. We have earlier proposed that the dihydropyridines reduce PCA by a single-step hydride-transfer mechanism and that such a mechanism is favored with oxidants which have two-electron capability.[5] The present results are indeed consistent with this proposal. Alternatively, one might propose that the PCA reaction proceeds through a rate-limiting electron transfer followed by a hydrogen atom transfer and attribute the isotope effect of ~ 1.24 (which is the value for k^{HH}/k^{HD} and $(k^{HH}/k^{DD})^{1/2}$ for the three compounds, **2** to **4** in Table I) to a secondary effect on the slow electron-transfer step. The observation that the positive charge in the nicotinamide moiety of the reductant is fully developed in the transition state[5] for the exergonic reaction[6] in eq 1 is more easily explained by this mechanism than a hydride-transfer mechanism. However, the isotope effect data in Table I do not support such a free radical mechanism unless the hydrogen atom transfer step has PIE of ~ 1. Furthermore, the SIE of ~ 1.24 is too large to be consistent with this mechanism; a secondary deuterium isotope effect of 1.0 $\pm$ 0.05 has been noted for the rate-limiting one-electron transfer from NADH during its oxidation by ferrocenium cation.[29]

The observed constancy of PIE's with various dihydronicotinamides and the agreement between kinetic and partition isotope effects (Table I) indicate that the multistep e^-, H^+, e^- mechanism where the proton transfer step is partly rate-limiting is unlikely. It is also unlikely that the PCA reaction proceeds by this multistep pathway with rate-determining proton transfer (which must occur directly) unless the dihydronicotinamide moiety in the transition state has virtually the radical cation structure.

If then the reaction does indeed proceed by a single-step hydride-transfer pathway, what is the structure of the transition state? There are reports[30] suggesting that hydride-transfer reactions must be facilitated by a bent transition state. This transition state is predicted to have low PIE's (2 to 3 range at 25 °C) independent of its symmetry.[31] Although the isotope effect data for the PCA reaction fit this prediction, we are suggesting a linear transition state[32] for the following reasons: (i) a wide range of PIE's up to a value greater than 11 has been observed for hydride-transfer reactions,[33] (ii) intermolecular hydride transfers appear to have linear transition states[34a] unless stereoelectronic effects dictate otherwise,[34b] and (iii) theoretical studies of the hydride transfer from cycloheptatriene and other compounds to cyclopropenium ion revealed that a linear or almost linear transition state is present in these reactions.[35]

When the low magnitude of PIE's for the PCA reaction is considered in conjunction with the unit Brønsted exponent for the reduction of PCA by 1-substituted 1,4-dihydronicotinamides, it appears that the reaction has a very product-like transition state. On the other hand, a $\Delta G°$ of -3.3 kcal/mol for the reaction in eq 1 suggests a nearly symmetric transition state, if the Hammond postulate is applied. However, it has become apparent that the a priori application of this postulate to bimolecular reactions in aqueous solution is incorrect;[36,37] consequently, the deduction of the transition-state structure from $\Delta G°$ of the PCA reaction is inappropriate. Although the nicotinamide moiety of the coenzyme is fully charged in the transition state, it is not known whether solvation of the transition state really resembles that of the products[37] and whether the in-flight hydrogen in the transition state is really completely transferred to the imino acid.[2]

Enzymatic Reaction. A $(PIE)_H$ of 3.80 for the PCA reaction (Table II) and an isotope effect of 4.1 for the oxidation of proline and proline-*2-d* by $NADP^+$ indicate that the hydride-transfer step is at least partly rate limiting in the enzymatic PCA–proline interconversion. These kinetic isotope effects produce a ratio of 0.93 $\pm$ 0.09, a value that is not significantly different from the corresponding equilibrium isotope effect of about 0.83.

The $(PIE)_D$ values are virtually independent of pH (over the range 6.2–9.0) even though the iminium ion binds and reacts with enzyme–NADPH about 15 times more effectively when a group, ZH^+, of pK 8.6 in the binary complex is unprotonated.[6] Presumably, both the rate and equilibrium constants for the dissociation of enzyme–NADPH–PCA are affected by the protonation state of Z. However, the observed $(PIE)_D$'s themselves remain unaltered with pH. These isotope effects also show only modest increases with decreasing temperature, an observation that is accounted for by the known exponential temperature dependence of isotope effects. Hence, it appears that there is no significant external commitment. It is still possible that there is internal commitment (such as a rate-contributing isomerization of the central complex), meaning that the reduction of PCA by E–NADH and E–[4,B-^{2}H]NADH complexes has an intrinsic PIE of at least 3.80.

In the absence of any significant reaction coorindate motion at the non-transferring hydrogen, eq 9 predicts that SIE ≤ 1.127 (the equilibrium secondary isotope effect value). The secondary isotope effect for deuterium transfer, $(SIE)_D$ of 1.06 (Table II), is indeed as predicted but that for hydrogen transfer, $(SIE)_H$ of 1.21, is much larger than 1.127. Such discrepancies have been noted in the nonenzymatic reduction of 4-cyano-2,6-dinitrobenzenesulfonate by NADH,[38] in the hydride transfer between NAD^+ analogues[39] and in a number of enzymatic reductions by NADH.[40] The secondary isotope effect data are best explained by assuming significant coupling between the in-place and in-flight hydrogens during the reaction coordinate motion.[38,40] However, model vibrational analysis calculations[41] show that the coupling produces calculated PIE's that are well below those observed and that a combination of α-hydrogen motion in the reaction coordinate and concomitant tunneling are required to explain *both* primary and secondary isotope effects whose magnitudes are of the size reported in Table II. We observe that deuteration at one site lowers the isotope effect at the other[39,42] by about 13%. This is as expected, since deuterium substitution should lead to an increase in the reduced mass of the reaction coordinate motion and hence a decrease in tunneling.[41] On the other hand, $(SIE)_H$ reflects both bonding changes of the nontransferred α-hydrogen and the extent to which this hydrogen contributes to the reaction coordinate motion.

Comparion of the Nonenzymatic and Enzymatic Reduction of PCA. The isotope effect data suggest that the nonenzymatic reaction has a very product-like transition state while the enzymatic reaction has a more symmetric one. Huskey and Showen[41] have proposed that the catalytic power of the dehydrogenases

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might be due, in part, to their ability to reduce the thickness of the reaction barriers, thereby promoting nuclear tunneling by hydrogen. However, the probability of tunneling is at a maximum only for a symmetric transition state,[43] and we indeed observe that the enzymatic reaction, in sharp contrast to the nonenzymatic reaction, has a more symmetric transition state.

Coenzyme Variation–Enzymatic PCA Reaction Rates. We have found that the reduction of the imino acid proceeds 10 times faster with E–NADH than with enzyme-reduced 3-acetylpyridine adenine dinucleotide (E–3-APADH) and 8 times[44] faster with NaDH than with 3-APADH. Since the substituent changes in the coenzymes are made close to the reaction site, we have abandoned the use of β values calculated from these reactivity ratios to describe the transition-state structures.[45] Instead, we have assumed that the relative changes in $\Delta G^{\ddagger}$ due to the nonelectronic effects of the amide and acetyl groups in the coenzymes are the same in the nonenzymatic and enzymatic reactions. In view of the transition state for the enzymatic reaction being less product-like than the nonenzymatic reaction, it is anomalous that the rates for the enzymatic reaction are more sensitive to coenzyme variation. We ascribe this anomaly to the unusually low reactivity of E–3-APADH and suggest[47] that 3-APADH, having no amide group, is unable to bind to the enzyme in a favorable fashion to derive entropic advantage of the subsequent step (which involves binding to PCA and reacting). There is in fact spectroscopic evidence[48] showing that there is interaction between the dihydropyridine ring and the enzyme in E–NADH but not in E–3-APADH. Furthermore, we have found that the enhanced reactivity of E–NADH over NADH itself in the PCA reaction is entirely due to a more favorable entropy of activation with the former reductant.[49]

Comparison to the Glutamate Reaction. The largest deuterium isotope effect observed for the glutamate dehydrogenase catalyzed reversible oxidative deamination of L-glutamate occurs in the initial burst produced by the enzyme–NADP$^+$–glutamate complex under single turnover conditions.[50] An isotope effect of 1.5 to 1.8 was measured[51] on this initial burst of the reaction with L-glutamate-*2-d*. If we assume that the isotope effect of 4.1 measured for the oxidation of proline and proline-*2-d* represents the intrinsic isotope effect for the glutamate oxidation then the hydride-transfer process must limit the initial burst rate by 16–26%. Clearly, the remaining contribution to rate-limitation is made by the forward commitment, involving partition ratios for steps up to the color-producing one[52] (λ_{max} 332 nm[51]).

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Registry No. [4-^{2}H]**1**, 17750-27-5; [4,4-^{2}H]**2**, 60172-94-3; [4,4-^{2}H]**3**, 96555-70-3; [4,4-^{2}H]**4**, 60764-22-9; PCA, 2139-03-9; deuterium, 7782-39-0; glutamate dehydrogenase, 9029-12-3.

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Specific Cleavage of Peptides Containing an Aspartic Acid (β-Hydroxamic Acid) Residue

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Abstract: Peptides containing the aspartyl β-hydroxamic acid residue are cleaved specifically at the carboxyl side of this residue at pH values ≥6. The cleavage occurs by attack of both the hydroxamic acid nitrogen and the hydroxamic acid oxygen to yield tetrahedral intermediates that, in the rate-limiting step, break down with cleavage of the peptide chain. In a competing reaction, the peptide nitrogen on the carboxyl side attacks the hydroxamate carbonyl to expel hydroxylamine and give an imide intermediate **28** (Scheme I). The cleavage yield reflects the relative efficiency of these two pathways. The extent of cleavage is dramatically increased in the presence of 1 M added hydroxylamine. The extent of cleavage is also increased significantly by phosphate buffers, but not by PIPES or imidazole buffers, in the absence of added hydroxylamine. The role of hydroxylamine in some cases may be to intercept the imide **28** or isoimide **33** (eq 8); but in at least two cases evidence is presented that hydroxylamine, like phosphate, may be acting as a general acid–base catalyst that selectively catalyzes the breakdown of tetrahedral intermediates **23** or **24** (Scheme I) leading to chain cleavage. Peptides containing glutamyl (γ-hydroxamic acid) residues are also cleaved, but at rates that are 20–40 times slower than those of the analogous aspartyl peptides. The results of this work completely rationalize in a mechanistic sense the hydroxylamine cleavage of Bornstein and Balian[6] and suggest a general method for the cleavage of peptides at aspartic acid residues.

About 1960 it was first recognized that peptides and proteins could be cleaved specifically with alkaline solutions of hydroxylamine under relatively mild conditions.[2] In 1969 Butler[3] and Bornstein[4] established that fragments of the α1 chain of rat

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