Secondary ^{15}N Isotope Effects on the Reactions Catalyzed by Alcohol and Formate Dehydrogenases†

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ABSTRACT: Secondary ^{15}N isotope effects at the N-1 position of 3-acetylpyridine adenine dinucleotide have been determined, by using the internal competition technique, for horse liver alcohol dehydrogenase (LADH) with cyclohexanol as a substrate and yeast formate dehydrogenase (FDH) with formate as a substrate. On the basis of less precise previous measurements of these ^{15}N isotope effects, the nicotinamide ring of NAD has been suggested to adopt a boat conformation with carbonium ion character at C-4 during hydride transfer [Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry 20*, 1817]. If this mechanism were valid, as N-1 becomes pyramidal an ^{15}N isotope effect of up to 2–3% would be observed. In the present study the equilibrium ^{15}N isotope effect for the reaction catalyzed by LADH was measured as 1.0042 ± 0.0007. The kinetic ^{15}N isotope effect for LADH catalysis was 0.9989 ± 0.0006 for cyclohexanol oxidation and 0.997 ± 0.002 for cyclohexanone reduction. The kinetic ^{15}N isotope effect for FDH catalysis was 1.004 ± 0.001. These values suggest that a significant ^{15}N kinetic isotope effect is not associated with hydride transfer for LADH and FDH. Thus, in contrast with the deformation mechanism previously postulated, the pyridine ring of the nucleotide apparently remains planar during these dehydrogenase reactions.

Liver alcohol dehydrogenase (LADH, EC 1.1.1.1) catalyzes the reversible oxidation of ethanol by NAD. The enzyme has been shown to utilize various primary and secondary alcohols, including cyclohexanol, as well as a number of NAD analogues (Wratten & Cleland, 1963; Hermes et al., 1984). The kinetic mechanism is partially random with NAD adding preferentially to the enzyme, followed by cyclohexanol. This ternary substrate complex rapidly converts to the corresponding ternary product complex followed by dissociation to the binary enzyme–NADH complex (Ainslie & Cleland, 1972).

In previous mechanistic studies of LADH, secondary ^{15}N isotope effects of 1.062 ± 0.007 for NAD reduction and 1.018 ± 0.005 for NADH oxidation were reported in which the equilibrium perturbation method and NAD labeled with ^{15}N in N-1 of the nicotinamide ring were used. The secondary ^{15}N equilibrium isotope effect was calculated from the perturbations as 1.044 ± 0.012 (Cook et al., 1981). On the basis of these results, it was postulated that hydride transfer catalyzed by dehydrogenases was facilitated by a distortion of the nicotinamide ring to produce a pyramidal nitrogen at N-1 and carbonium ion character at C-4.

Yeast formate dehydrogenase (FDH, EC 1.2.1.2) catalyzes the irreversible oxidation of formate by NAD to give CO_2 and NADH. FDH has an ordered kinetic mechanism in which NAD adds first to the enzyme followed by formate (Blanchard & Cleland, 1980). A large secondary ^{15}N kinetic isotope effect of 1.07 ± 0.04 measured for FDH according to the method of direct comparison by Hermes et al. (1984) was proposed to support the geometric deformation mechanism for hydride transfer.

Subsequent experiments have suggested that the values obtained by Cook et al. (1981) may not be correct. The ^{15}N kinetic and equilibrium isotope effects for the reversible uncatalyzed reaction of cyanide with NAD were determined by Burgner et al. (1987), with the $^{15}K_{eq}$ value being 1.004 ± 0.002. By making quantum-mechanical calculations for NAD and NADH, Huskey (1985) proposed a value of 1.008 for the $^{15}K_{eq}$ for dehydrogenases. A preliminary ^{15}N equilibrium isotope effect at N-1 of NAD has been obtained with yeast alcohol dehydrogenase using isopropanol as a substrate (P. M. Weiss, & Hermes, J. D., unpublished observations). After chemical and isotopic equilibrium had been reached, the residual NAD was decomposed to 2-hydroxy-3-pyridinecarboxaldehyde and ADP-ribosylamine with a strong base. Guilbert and Johnson (1977) claimed that at high pH this reaction produced solely these products and that the ammonia generated from the ADP-ribosylamine came from N-1 and not from the exocyclic nitrogen. It is not clear, however, whether some ammonia also comes from the side chain. The tentative value for the ^{15}N equilbrium isotope effect of 1.0009 ± 0.0004 determined by Weiss and Hermes using isotope-ratio mass spectrometric analysis of this ammonia also suggests that the previous values for LADH-catalyzed hydride transfer were too high.

In order to determine the mechanism of hydride transfer, the experiments that led to the seemingly anomalous ^{15}N isotope effects at N-1 obtained by Cook et al. (1981) have been repeated with the more sensitive method of internal competition using the 0.37% natural abundance of ^{15}N in the nucleotide as the label. The ^{15}N secondary equilibrium and kinetic isotope effects for LADH and the kinetic isotope effect for FDH have been measured with 3-acetylpyridine adenine dinucleotide (Acpyr-NAD), an analogue of NAD in which the amide moiety of NAD has been replaced with an acetyl group. The isotope effects were determined with this analogue since acetylpyridine contains only one nitrogen at the N-1 position of the pyridine ring and can be degraded easily to ammonia, which is subsequently oxidized to nitrogen gas for analysis in an isotope-ratio mass spectrometer.

Materials and Methods

Nomenclature. The nomenclature used is that of Northrop (1977), in which an isotope effect on kinetic or thermodynamic parameters is indicated by leading superscripts. Thus $^{15}K_{eq}$ is the ^{15}N equilibrium isotope effect ($K_{eq\ N\text{-}14}/K_{eq\ N\text{-}15}$), and

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$^{15}(V/K)$ is the ^{15}N kinetic isotope effect on the parameter V/K.

Materials. 3-Acetylpyridine adenine dinucleotide, glucose-6-phosphate, oxaloacetate, glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, type XXIV), pig heart malic dehydrogenase, rabbit lactate dehydrogenase, horse liver alcohol dehydrogenase, nucleotide pyrophosphatase (type III, *Crotalus atrox* venom), and ammonia color reagent were from Sigma. Calf intestine alkaline phosphatase and yeast formate dehydrogenase were from Boehringer Mannheim. Sephadex A-25 resin was from Pharmacia. Reverse-phase silica gel resin was prepared by the method of Kuhler and Lindsten (1983).

Instrumentation. A Beckman DU spectrophotometer was used to follow the reactions. The molecular nitrogen samples were isolated according to Hermes et al. (1985), by using a vacuum system equipped with mechanical and diffusion pumps able to pump to less than 10^{-7} torr. Samples to be analyzed contained 20–75 μmol of molecular nitrogen. A Finnigan MAT 251 isotope-ratio mass spectrometer was used to measure the isotopic content of the nitrogen produced in the reactions. The isotopic ratios of the peaks at m/e 29 and 28 were obtained as delta values [δ = 1000(isotopic ratio of sample/isotopic ratio of standard) − 1], and thus $R = \delta/1000 + 1$. Equilibrium isotope effects were calculated from eq 1 when the mass ratio in the residual reactant at equilibrium ($R_{s\,eq}$) was compared with that in the initial reactant (R_0) and from eq 2 when the mass ratio in the product at equilibrium ($R_{p\,eq}$) was compared with R_0. In these equations x_i = [product]/[residual substrate] at equilibrium.

Kinetic isotope effects were calculated from eq 3 when mass ratios of the initial and residual substrates were compared and from eq 4 when mass ratios of the product and the initial substrate was used. In these equations, f is the fractional reaction (that is, the product formed relative to the initial substrate concentration).

$$^{15}K_{eq} = x_i/[(1 + x_i)(R_0/R_{s\,eq}) - 1] \quad (1)$$

$$^{15}K_{eq} = R_0/R_{p\,eq} + x_i[(R_0/R_{p\,eq}) - 1] \quad (2)$$

$$^{15}(V/K) = \log (1 - f)/\log [(1 - f)(R_s/R_0)] \quad (3)$$

$$^{15}(V/K) = \log (1 - f)/\log [1 - f(R_p/R_0)] \quad (4)$$

Determination of the ^{15}N Equilibrium Isotope Effect. All experiments were performed at room temperature, and for each, two controls were run: one with the enzyme but lacking substrates (to be sure no endogenous nucleotides were present) and one without the enzyme but containing reactants at half of their normal concentrations (to be sure that the mass ratio in the starting substrate after isolation was the same as that before addition to the reaction mixture). Acpyr-NAD was purified in 600-μmol batches on a 2.5 × 30 cm column containing 143 mL of Sephadex A-25 resin and monitored at 254 nm with a UV monitor. Acpyr-NAD was dissolved in 2 mL of water and the pH adjusted to 3 prior to loading the column. The column was flushed with 150 mL of water, and Acpyr-NAD was eluted with 50 mM NaCl and rotary evaporated to 50 mL. The concentration was determined enzymatically at 363 nm by a glucose-6-phosphate dehydrogenase assay.

The ^{15}N equilibrium isotope effect studies were conducted with LADH and cyclohexanol, for which the equilibrium constant is

$$K_{eq} = \frac{[\text{Acpyr-NADH}][\text{cyclohexanone}][H^+]}{[\text{Acpyr-NAD}][\text{cyclohexanol}]} = 7.65 \times 10^{-8}\ \text{M} \quad (5)$$

The reaction was initiated by adding 140 units of LADH to 2 mM Acpyr-NAD, 4 mM cyclohexanol, and 16 mM cyclohexanone in 100 mL of water. The pH was initially adjusted to 8.3 and was held there by the addition of 0.25 N NaOH for 2.5 h until chemical equilibrium was attained, as determined by the change in A_{363}. The reaction was then left to reach isotopic equilibrium over the next 24 h. The protein was then removed by using a YM30 membrane in an Amicon stirred cell concentrator, and the filtrate was loaded in a volume of 1 L onto the DEAE Sephadex A-25 column in the chloride form. Once loaded, the column was flushed with 150 mL of water, and Acpyr-NAD was eluted with 50 mM NaCL, while Acpyr-NADH was eluted with 200 mM NaCl adjusted to pH 8.6 with NaOH. The fractions containing Acpyr-NAD and Acpyr-NADH were separately pooled, and the concentrations were determined enzymatically with glucose-6-phosphate or malate dehydrogenase assays with glucose-6-phosphate or oxaloacetate as substrates. To increase the stability of the nucleotides, the pH of the Acpyr-NAD sample was lowered to 3 while that of Acpyr-NADH was raised to 9.

The Acpyr-NADH was then completely oxidized to Acpyr-NAD with 20 mM pyruvate and 9000 units of lactate dehydrogenase at pH 7.5 for 6 h in a 100-mL volume. Once oxidation was complete, the pH was lowered to 3 and the protein was removed by Amicon filtration.

Acpyr-NAD samples were reduced to 20 mL by rotary evaporation and degraded in the presence of 2 mM $MgCl_2$ and 0.2 mM $ZnCl_2$ to adenosine, 3-acetylpyridine-ribose, and phosphate by 200 units of alkaline phosphatase and 27 units of nucleotide pyrophosphatase per sample. The pH was adjusted to 7.5 before the addition of the enzymes and held between 7.5 and 8.5 by the addition of NaOH during the reaction. Aliquots were removed during the reaction and assayed for phosphate with the Malachite green assay (Carter & Karl, 1982). After ~15 h degradation was complete, and the reaction was quenched to pH 3 with 3 N sulfuric acid. The samples were rotary evaporated to 1–3 mL and loaded on a 6 × 20 cm column containing 190 mL of reverse-phase silica gel preequilibrated with degassed water. The flow rate was adjusted with N_2 at <5 psi, and the columns were flushed with 100 mL of water prior to elution of acetylpyridine-ribose with 5% methanol and adenosine with 100% methanol. The purified acetylpyridine-ribose fractions were identified by their characteristic absorption spectrum from 230–400 nm (λ_{max} = 262 nm). The peak fractions were pooled, concentrated by rotary evaporation to 1–2 mL, and digested by the Kjeldahl procedure for 12–18 h. The ammonia was isolated by alkaline steam distillation (Burris, 1957). Sigma ammonia color reagent was used to quantify the ammonia by the Nessler assay. The purified ammonia was oxidized to N_2 in vacuo with 2 mL of hypobromite solution in a Y tube. After 15 min the reaction was quenched by freezing in liquid nitrogen, and the N_2 sample was transferred from the Y tube under vacuum (10^{-7}–10^{-8} torr) and absorbed onto 4-Å molecular sieves, 8–12 mesh, at liquid nitrogen temperature prior to analysis in the isotope-ratio mass spectrometer (Hermes et al., 1985).

Determination of the ^{15}N Kinetic Isotope Effect with LADH and Cyclohexanol. The ^{15}N isotope effect studies in the forward direction with Acpyr-NAD as a substrate were conducted as follows. To a 20-mL unbuffered reaction mixture at pH 8.3 containing 10 mM Acpyr-NAD and 15–25 units of LADH was added 0.2 mM cyclohexanol. The concentration of cyclohexanol was held constant below its K_m of 0.56 mM throughout the 20-min reaction by adding 0.65 μmol every 10 s while the pH was held constant by the addition of 0.1 N NaOH.

Table I: ^{15}N Equilibrium Isotope Effects Determined with Liver Alcohol Dehydrogenase and Cyclohexanol and Cyclohexanone[a]

expt no.	[Acpyr-NADH]$_{eq}$ / [Acpyr-NAD]$_{eq}$	δ corresponding to $R_{p\,eq}$	δ corresponding to $R_{s\,eq}$	δ corresponding to R_0	$^{15}K_{eq}$
1	0.232	12.431		16.761	1.0053
2	1.000	14.719		15.647	1.0018
3	0.973	14.660		17.488	1.0055
4	0.963		17.097	14.494	1.0052
		12.733		14.494	1.0034
av					1.0042 ± 0.0007

[a] Reactions were brought to equilibrium at pH 8.3, 25 °C. Isotope effects were calculated from eqs 1 or 2 by using R values derived from the δ values tabulated above.

The reaction was monitored by using a peristaltic pump to circulate the reaction mixture from the beaker through a UV monitor at 363 nm and back into the beaker. A linear change in absorbance over time was observed, consistent with a constant level of cyclohexanol. After 20 min the reaction was quenched by lowering the pH to 3 with 3 N HCl, and the fraction of reaction was determined enzymatically from the residual nucleotides by using a glucose-6-phosphate dehydrogenase assay. The oxidized and reduced nucleotides were then separated and the oxidized nucleotide degraded for generation of N_2 as described above.

Determination of the ^{15}N Kinetic Isotope Effects with LADH and Cyclohexanone. Acpyr-NADH was synthesized enzymatically from purified Acpyr-NAD with 40 mM glucose-6-phosphate and 112 units of glucose-6-phosphate dehydrogenase. The resulting Acpyr-NADH was purified by chromatography with the DEAE Sephadex A-25 column in the chloride form. The Acpyr-NADH eluted in 200 mM NaCl was adjusted to pH 8.6, and peak fractions were combined and rotary evaporated to 20 mL. The kinetic isotope effect experiments were carried out in a 10-mL volume containing 16 mM purified Acpyr-NADH and 56 units of LADH at pH 7. The reaction was initiated with 1 mM cyclohexanone, and its concentration was held constant at 1 mM (below the K_m of 4 mM) by the addition of cyclohexanone at a constant rate of 0.15 mM per 30 s. Aliquots were removed every minute, and the linear decrease in A_{363} indicated a constant cyclohexanone concentration during the reaction. The oxidized and reduced nucleotides were again separated, and N_2 was generated from the oxidized nucleotide product as described above.

Determination of the ^{15}N Kinetic Isotope Effect with Formate Dehydrogenase. These studies with formate dehydrogenase were run below the K_m of formate (335 mM), with the same apparatus described for LADH. To an unbuffered solution at pH 7.6 containing 9 mM Acpyr-NAD and 90 units of FDH in 20 mL was added 30 mM potassium formate. The pH was held at 7.6 throughout the 1.5–5-h reaction time. The oxidized and reduced nucleotides were separated, and N_2 was generated from the residual Acpyr-NAD as described above.

Results and Discussion

^{15}N Equilibrium Isotope Effect. The $^{15}K_{eq}$ isotope effect at pH 8.3 with natural abundance labeled N-1 of Acpyr-NAD with LADH and cyclohexanol and cyclohexanone was 1.0042 ± 0.0007 (Table I). This value is consistent with both the cyanide-adduct studies of Burgner et al. (1987) and the calculations of Huskey (1985). The modest value measured in the present study indicates that the bond order at N-1 changes only slightly and is closer to 4 than 3 in the reduced nucleotide

Table II: ^{15}N Kinetic Isotope Effects Determined with Liver Alcohol Dehydrogenase and Cyclohexanol as a Substrate[a]

expt no.	fract. reaction	δ corresponding to R_s	δ corresponding to R_0	$^{15}(V/K)$
1	0.371	15.826	16.917	0.9976
2	0.742	15.986	15.885	1.0001
3	0.454	16.228	16.316	0.9998
4	0.293	14.794	15.334	0.9982
av				0.9989 ± 0.0006

[a] Reactions were run at pH 8.3, 25 °C, with cyclohexanol held constant at 0.2 mM. Isotope effects were calculated from eq 3 by using R values derived from the δ values tabulated above.

Table III: ^{15}N Kinetic Isotope Effects Determined with Liver Alcohol Dehydrogenase and Cyclohexanone as a Substrate[a]

expt no.	fract. reaction	δ corresponding to R_p	δ corresponding to R_0	$^{15}(V/K)$
1	0.360	12.639	13.593	1.0012
2	0.285	14.813	11.543	0.9960
3	0.328	15.785	11.543	0.9949
av				0.997 ± 0.002

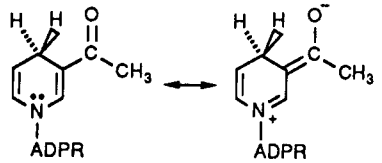
[a] Reactions were run at pH 7.0, 25 °C, with cyclohexanone held constant at 1 mM. Isotope effects were calculated from eq 4 by using R values derived from the δ values tabulated above.

Table IV: ^{15}N Kinetic Isotope Effects Determined with Formate Dehydrogenase and Formate as a Substrate[a]

expt no.	fract. reaction	δ corresponding to R_s	δ corresponding to R_0	$^{15}(V/K)$
1	0.174	15.329	14.478	1.0044
2	0.282	16.573	14.478	1.0063
3	0.243	17.154	16.203	1.0034
4	0.326	14.853	14.314	1.0013
av				1.004 ± 0.001

[a] Reactions were run at pH 7.6, 25 °C, with formate initially at 30 mM. Isotope effects were calculated from eq 3 by using R values derived from the δ values tabulated above.

as a result of a strong contribution of the dipolar form of Acpyr-NAD



This interpretation is consistent with crystallographic studies in which the measured N-1 to C-2 bond lengths for NAD and NADH analogues do not differ substantially (Karle, 1961; Johnson et al., 1973).

The $^{15}K_{eq}$ value of 1.044 reported by Cook et al. (1981) was calculated from four points generated from the change in perturbation size with the varying ratio of the nucleotide concentrations. The one point that differed appreciably from the other three and thus effectively determined the value may have been in error.

^{15}N Kinetic Isotope Effects. With LADH $^{15}(V/K)$ in the direction of cyclohexanol oxidation was 0.9989 ± 0.0006 (Table II) and in the direction of cyclohexanone reduction was 0.9974 ± 0.0019 (Table III). With FDH at pH 7.6 the ^{15}N isotope effect for the oxidation of formate was 1.004 ± 0.001 (Table IV). Isotope effects on the FDH reaction have been shown to be the intrinsic ones on the bond-breaking steps (Blanchard & Cleland, 1980), and the level of formate used in our experiments was low enough that no correction is needed

for an external commitment induced by formate. The LADH reaction with cyclohexanol and cyclohexanone has been shown by Cook and Cleland (1981a,b) to involve a largely ordered kinetic mechanism; and in such a case isotope effects measured for the first substrate to add (Acpyr-NAD or Acpyr-NADH) are eliminated by saturation with the second substrate (Cook & Cleland, 1981a). The level of the second substrate was kept as low as possible in our experiments, but the observed isotope effects need to be corrected for the effect of the second substrate by eq 6, where B is the concentration of cyclohexanol or cyclohexanone, K_{ia} and K_a are dissociation and Michaelis constants for the nucleotide, and K_b is the Michaelis constant for the alcohol or ketone. Substitution of the observed isotope effects in eq 6 gives corrected $^{15}(V/K_b)$ values of 0.9979 in the forward direction with Acpyr-NAD and 0.9961 in the reverse direction.

$$^{15}(V/K_b) = 1 + [(\text{obs. I.E.}) - 1]\,[1 + K_aB/(K_{ia}K_b)] \quad (6)$$

Since there is an internal commitment of 2.5 with cyclohexanol as a substrate (Cook et al., 1981), the intrinsic isotope effects in the forward and reverse reactions can be calculated from eqs 7 and 8 on the assumption that only the hydride-transfer step would show an ^{15}N isotope effect at N-1 of the acetylpyridine ring:

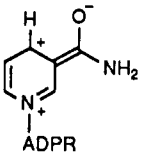
$$^{15}(V/K_{\text{cyclohexanol}}) = \frac{^{15}k_{\text{forward}} + 2.5}{1 + 2.5} \quad (7)$$

$$^{15}(V/K_{\text{cyclohexanone}}) = \frac{^{15}k_{\text{reverse}} + 2.5\,^{15}K_{eq}}{1 + 2.5} \quad (8)$$

$^{15}K_{eq}$ in eq 8 is the ^{15}N equilibrium isotope effect in the reverse reaction (0.996 measured experimentally in the present work). Application of these equations gives 0.993 for $^{15}k_{\text{forward}}$ from eq 7 and 0.996 for $^{15}k_{\text{reverse}}$ from eq 8. If $^{15}K_{eq}$ in eq 8 is taken as 0.998, which is the ratio of experimental $^{15}(V/K_b)$ values, the calculated $^{15}k_{\text{reverse}}$ value is 0.991. Since the ratio of $^{15}k_{\text{reverse}}/^{15}k_{\text{forward}}$ must equal $^{15}K_{eq}$, either $^{15}K_{eq}$ is truly 1.002 or one of the $^{15}(V/K_b)$ values is slightly in error. We believe the most likely interpretation of the data is that the observed isotope effect in the forward direction should be unity, in which case $^{15}k_{\text{forward}}$ would be unity. The value for $^{15}k_{\text{reverse}}$ would be 0.996, and the ratio would be equal to the measured $^{15}K_{eq}$ value of 1.004.

Acpyr-NAD vs NAD. The use of Acpyr-NAD as a model for hydride transfer to NAD should be valid and should not alter the isotope effects significantly. The long wavelength absorbance for NADH caused by the dipolar resonance form is present in Acpyr-NADH as well as in NADH, although at a slightly longer wavelength. Acpyr-NAD has a more positive redox potential than NAD (−0.258 vs −0.32 V), and the transition states for FDH (Blanchard & Cleland, 1980; Hermes et al., 1984) and LADH (Scharschmidt et al., 1984) are earlier with this nucleotide than with NAD. A slight change in isotope effects might thus be expected, but this would not account for the order of magnitude differences between the values reported by Cook et al. (1981) and those determined in the present work.

Catalysis of Hydride Transfer. The present work shows that N-1 does not undergo an appreciable decrease in bond order during hydride transfer and thus that the pyridine ring of Acpyr-NAD (and presumably of NAD) is not distorted into a boat, which would force a decrease in the bond order at N-1 to 3. How then is hydride transfer catalyzed? LeReau et al. (1989) have shown a deuterium isotope effect at C-4 of the nicotinamide ring upon the binding of NAD to lactate dehydrogenase of ~10%, which argues for the development of carbonium ion character at C-4. The 200–300-fold faster reaction of cyanide with the binary enzyme–NAD complex than with free NAD supports this view (Gerlach et al., 1965; Burgner & Ray, 1984). If carbonium ion character appears at C-4 without changing the bond order to N-1, the enzyme must be stabilizing, as the result of hydrogen-bonding interactions with the side chain, a dipolar form such as



It would be interesting to see whether there is an ^{18}O isotope effect in the side chain of NAD during dehydrogenase reactions!

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