Isotope Effects on Binding of NAD^+ to Lactate Dehydrogenase†

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ABSTRACT: The isotope effect on binding [4-^{2}H]NAD^+ and [4-^{3}H]NAD^+ to lactate dehydrogenase has been shown to be 1.10 ± 0.03 by whole molecule isotope ratio mass spectrometry and 1.085 ± 0.01 by $^3H/^{14}C$ scintillation counting. These values demonstrate that specific interactions of the nicotinamide ring with the enzyme make the C–H bond at C-4 less stiff in the binary complex.

The binding of isotopically labeled molecules to enzyme active sites has commonly been assumed to be unaffected by the isotopic label (Cleland, 1987). This assumption has proven to be acceptable when one considers primary deuterium isotope effects where potential isotope effects of 3–10 on bond-forming and bond-breaking reactions are an order of magnitude larger than the secondary deuterium isotope effects that result from changes in the vibrational energy potentials of the substituted hydrogens (Hartshorn & Shiner, 1972). Enzymologists have resorted to observation of smaller secondary isotope effects while retaining the assumption that the isotope effect on binding is negligible compared to the effect that occurs during the catalytic chemical reorganization (Mentch et al., 1987; Hermes et al., 1984).

The assumption that there is no isotope effect on binding is inconsistent with a second theme of enzymology: that enzymes are designed to bind more tightly to the transition state (Pauling, 1946; Wolfenden, 1972). A corollary of this theme is the concept that some of the potential free energy of binding may be used to distort the substrate toward the transition-state structure (Jencks, 1975; Fersht, 1987). This change in substrate conformation or electronic configuration can in opportune cases be observed. Spectroscopic studies (Ross et al., 1982) and NMR studies (Lee et al., 1974; Levy et al., 1983; Oberfrank et al., 1984) on NADH and NAD^+ binding to dehydrogenases have confirmed that the environment and conformation of the bound nucleotide are significantly different from the solution structure. X-ray crystallography can determine conformational properties of the nucleotide including estimates of bond angles, but the resolution is rarely sufficient to establish whether a specific bond has been distorted significantly from the local minimum of the molecule free in solution (Grau, 1982). Only in vibrational and electronic spectroscopy can small deviations in electron distribution be observed. FT-IR studies have shown that the carbonyl stretches for dihydroxyacetone phosphate and oxalacetate are red shifted on binding to triosephosphate isomerase (Belasco & Knowles, 1980) and citrate synthase (Kurz & Drysdale, 1987), respectively. These observed changes in carbonyl vibrational frequency would be manifested as small but real and calculable isotope effects on binding if either the carbonyl oxygen or carbon were substituted with a heavier isotope. The heavier isotope would be preferentially enriched in the compound with the bond that has a greater zero point energy from all of the contributing vibrations. Raman spectroscopy of NADH and NAD^+ with horse liver alcohol dehydrogenase has detected that significant vibrational changes occur on binding of the coenzyme (Chen et al., 1987), but these vibrations have not been associated with specific vibrational modes. If isotope effects on binding could be measured precisely enough, it would provide a general method for detecting strained intermediates. Previous attempts at measuring isotope effects on substrates binding to enzymes have either led to results that are too large to be realistically accommodated by current theory (Bush et al., 1971) or not significantly different from 1.00 (deJuan & Taylor, 1976; Andersson et al., 1985).

The phenomenal change in equilibrium constant for the oxidation of lactate by NAD^+, increasing from 2.5×10^{-5} free in solution at pH 7.0 to greater than 1.0 for the substrates bound to lactate dehydrogenase (LDH)[1] (Schwert et al., 1967; Nambiar et al., 1983), attracted our attention. This 5 order of magnitude change in the equilibrium constant implies that lactate dehydrogenase must destabilize the NAD^+–lactate complex by nearly 7 kcal/mol relative to the NADH–pyruvate complex. One way to accomplish this is to remove electron density from the nicotinamide ring by specific interactions with the enzyme, thus destabilizing NAD^+ relative to NADH while making it a better oxidant. This increase in positive charge would be reflected in the vibrational frequency of the C-4 hydrogen bond and result in an equilibrium isotope effect on the association constant for NAD^+ with the enzyme. In this paper we present two sensitive competitive methods for measuring small isotope effects on binding. The results of these experiments indicate that there is a significant isotope effect on the association of [4-^{2}H]NAD^+ and [4-^{3}H]NAD^+ with LDH.

Materials and Methods

Materials. Hexokinase (bakers' yeast), glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), LDH (porcine heart), and pyruvate were purchased from Sigma. Nearly carrier free [1-^{3}H]glucose (25 Ci/mmol) was purchased from ICN, and 98% [1-^{2}H]glucose was purchased from Omicron Biochemicals. Nicotinamide [U-^{14}C]adenine dinucleotide (303 mCi/mmol) was purchased from Amersham. All other chemicals were of reagent grade and were used without further purification.

Synthesis of [4-^{3}H]NAD^+. A solution of 30 mM Na^+-TAPS buffer (pH 7.8) containing 12 mM magnesium acetate, 3.2 μM [1-^{3}H]glucose, 300 μM ATP, and 3 units of hexokinase in a total volume of 750 μL catalyzed the formation of [1-^{3}H]glucose 6-phosphate. NAD^+ was then added to 0.30–0.35 mM with 2 units of glucose-6-phosphate dehydrogenase to

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[1] Abbreviations: LDH, lactate dehydrogenase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PFK, perfluorokerosene; SIM, selected ion monitoring; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TIC, total ion current.

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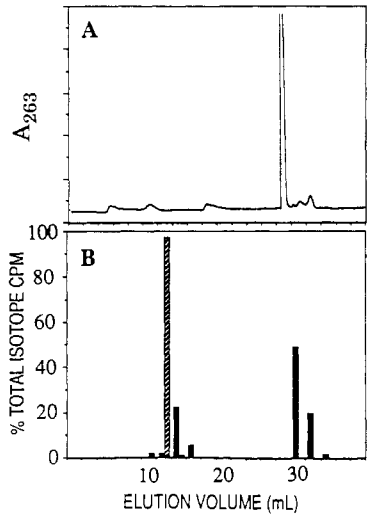


FIGURE 1: (A) HPLC elution of NAD^+ monitored by the absorbance at 263 nm. (B) Elution of [4-^{3}H]NAD^+ (solid bars) and [U-^{14}C]-NAD^+ (hatched bars) under identical conditions aftter 48 h at pH 12.0.

form [4-^{3}H]NADH, which was then oxidized in situ by addition of 0.1 mg of pyruvate and 2 units of LDH. The addition of tritium to the *pro-4S* position and subsequent removal of the *pro-4R* protium of NADH leaves a solution of 95–110 mCi/mmol [4-^{3}H]NAD^+. After 30 min the final solution was quenched with formic acid to pH 3. For the binding experiment [^{14}C]NAD^+ was added to the synthesis mixture prior to isolation.

NAD^+ and Nicotinamide Isolation. NAD^+ and nicotinamide were isolated by HPLC on a 0.46 × 25 cm octadecylsilyl Econosphere (Alltech) column equilibrated with 30% 0.2 M ammonium formate (pH 2.4) and 70% H_2O. For the NAD^+ isolation, 20 min after the injection a 5-min linear gradient to 20% methanol, 50% H_2O, and 30% ammonium formate buffer was run. NAD^+ eluted in a single sharp peak at 8.0 min after the gradient began (28.0 min after injection; Figure 1). Peaks were detected at 263 nm and by scintillation counting. Nicotinamide was isolated by running identical initial conditions for 10 min followed by a 10-min linear gradient to 100% methanol. The nicotinamide eluted 20 min after injection. The fraction containing the NAD^+ or nicotinamide was dried directly in a Savant Speed Vac concentrator under low heat and high vacuum.

Synthesis of [4-^{2}H]NAD^+. The same procedure was used as above by substituting [1-^{2}H]glucose and increasing the scale. A total volume of 2 mL of 30 mM Na^+-TAPS buffer (pH 7.8) was prepared containing 30 mM magnesium acetate, 8.2 mM [1-^{2}H]glucose, and 7.5 mM NAD^+ to make (4*S*)-[4-^{2}H]-NADH. Pyruvate (35 μmol) and 10 units of LDH were added to oxidize the (4*S*)-[4-^{2}H]NADH, leaving [4-^{2}H]NAD^+. The [4-^{2}H]NAD^+ was isolated by HPLC (vide supra) in five separate injections. The samples were dried and stored at −20 °C until used.

LDH solutions were desalted by passing them through a 3-mL G-50 fine Sephadex centrifuge column (Penefsky, 1977) equilibrated and centrifuged with 30 mM Na^+-MOPS buffer (pH 7.8).

[4-^{3}H]NAD^+ Equilibrium Isotope Effect on Association. One milliliter of a 0.80–1.0 mM LDH solution was added to the purified and dried [^{14}C/^{3}H]NAD^+, and the solution was allowed to equilibrate for 5–10 min before the sample was added to one side of the equilibrium dialysis cell. The dialysis cell contained a total volume of 2 mL, separated into two 1-mL halves by a Spectra/Por 2 12 000–14 000 molecular weight cutoff dialysis membrane. The other side of the cell was filled with 1 mL of the 30 mM Na^+-MOPS buffer solution (pH 7.8). The dialysis cell was allowed to equilibrate for 5–8 h, after which time NAD^+ assays showed no further change in concentration on either side of the dialysis cell. To demonstrate complete equilibration, two determinations of the isotope effect were made by adding the NAD^+ to the enzyme-free side of the dialysis cell (experiments 7 and 8, Table I). Samples from each side of the dialysis cell were removed and separately titrated to pH 3 with formic acid. After the addition of 10 mM ammonium sulfate the denatured enzyme was pelleted in an Eppendorf 5414 tabletop centrifuge. NAD^+ was isolated from any decomposition products by HPLC under the same conditions as described above. Special care was taken to manually collect the entire NAD^+ peak to avoid the possibility of isotopic fractionation on the column or contamination by nicotinamide. The concentration of NAD^+ on both sides of the dialysis membrane was determined by adding a 25-μL aliquot to an assay solution containing 1 mM glucose 6-phosphate and glucose-6-phosphate dehydrogenase and following the increase in absorption at 340 nm (ϵ_{340} = 6220 cm^{-1} M^{-1}).

Scintillation Counting. Two 500-μL volumes of each sample were placed in scintillation vials to which 15 mL of Ecolume scintillation fluid was added. Each vial was counted on a Packard Tri-Carb 3330 scintillation spectrometer more than 5 times for 1–10 min each to determine the total ^{14}C/^{3}H NAD^+ ratios. The ^{14}C window was set so that less than 1% of the counts of a standard tritium sample was detected in that window. The ^{3}H window was set so that about 12% of the counts of a ^{14}C sample were detected by the ^{3}H channel. For an accurate correction in the ^{3}H channel, a standard sample of [^{14}C]NAD^+ was counted each time a set of [^{14}C/^{3}H]NAD^+ samples was counted.

Calculations. The ^{3}H isotope effect for binding can be determined by

$$^{T}K_A = R_B/R_F \quad (1)$$

where K_A is the equilibrium constant for NAD^+ binding to LDH. A normal isotope effect on the association constant, i.e., $K_A > 1$, indicates that the enzyme preferentially binds the lighter isotope, and consequently the isotopically substituted bond is less stiff in the enzyme substrate complex. R_B is ^{14}C/^{3}H bound to the enzyme and R_F is ^{14}C/^{3}H free in solution. R_F is measured directly from the sample obtained from the enzyme-free side of the dialysis cell, but R_B must be calculated from

$$R_B = R_M/f_B + [(f_B - 1)/f_B]R_F \quad (2)$$

where R_M is ^{14}C/^{3}H actually measured for the NAD^+ obtained from the side of the dialysis cell that contained LDH. The fractions of NAD^+ bound and free, f_B and f_F, respectively, were determined by NAD^+ assays of 25-μL aliquots obtained from each side of the dialysis cell prior to removal of the solutions from the dialysis cell.

[4-^{2}H]NAD^+ Isotope Effect on Binding. The equilibrium dialysis cell used for the [4-^{2}H]NAD^+ experiments contained a 0.3–0.4 mM amount of a 1:1 mixture of [^{2}H]-NAD^+–[^{1}H]NAD^+ in 1 mL of the LDH solution on one side and 4 mL of buffer solution on the other. All other conditions were identical with the [4-^{3}H]NAD^+ experiments.

At the end of the equilibration time for the cell, both sides were removed and separately titrated to pH 12 with Na_3PO_4 to hydrolyze the glycosidic bond of the NAD^+, leaving nicotinamide. Johnson et al. (1988) have shown that higher pH does not accelerate the hydrolysis but does promote the for-

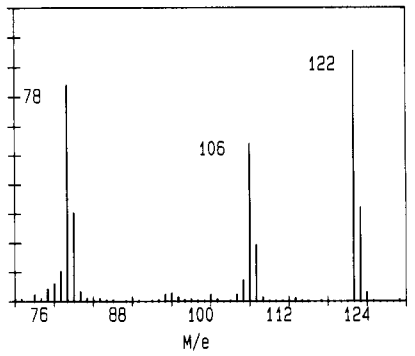


FIGURE 2: Low-resolution mass spectrum of a 70:30 mixture of [4-^{1}H]NAD$^+$–[4-^{2}H]NAD$^+$ indicating the parent ion is the base peak, and M − 1 peak is absent, and the two major fragments retain the C-4 hydrogen.

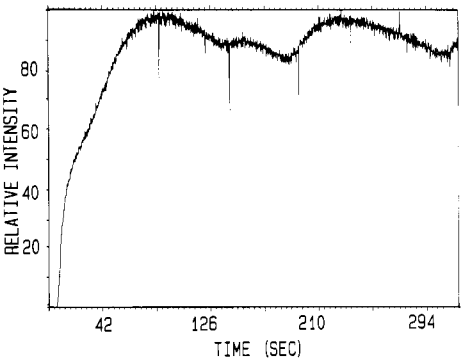


FIGURE 3: Total ion current versus time for a typical niacinamide isotope ratio determination using the SIM acquisition parameters described under Materials and Methods. The calculated isotope ratios for each 42-s interval (200 measurements) after the maximum signal was reached varied by less than 1%.

mation of 2-hydroxypyridonecarboxaldehyde. A control mixture of both [4-^{3}H]NAD$^+$ and [*adenyl*-U-^{14}C]NAD$^+$ was allowed to remain at this high pH for 48 h and was then titrated to pH 2.0; products were then isolated with the NAD$^+$ procedure (vide supra) to ensure that no exchange from C-4 occurred during the hydrolysis or that radioactive decomposition products did not coelute with the NAD$^+$ during its HPLC isolation. The tritium from the NAD$^+$ hydrolysis elutes in two fractions, a small peak at 15 min and a large fraction at 31 min. The large fraction coelutes with authentic [4-^{3}H]nicotinamide. The small fraction is presumably nicotinic acid (Johnson et al., 1988). All of the ^{14}C eluted at 14 min. No radioactivity was found in the first 10 min of the HPLC isolation, confirming that no exchange of the C-4 hydrogen with solvent took place during the hydrolysis or subsequent isolation (Figure 1).

The nicotinamide-containing fractions were dried directly as described above. Dried nicotinamide samples were dissolved in 25 μL of ethyl acetate (analytical grade) and divided into five glass capillary tubes for the direct probe of the mass spectrometer. The solvent was removed under vacuum in the Savant concentrator.

$^2H/^1H$ NAD$^+$ Analysis. In the EI$^+$ mass spectrum of nicotinamide the base peak is the parent ion and the major fragments are loss of NH_2 and $CONH_2$, which should not be affected by substitution at C-4. The spectrum of a 70:30 mixture of [4-^{1}H] nicotinamide–[4-^{2}H]nicotinamide is shown in Figure 2, confirming the presence of the C-4 hydrogen in these peaks and demonstrating the absence of an M − 1 peak. A Kratos MS80RFA mass spectrometer interfaced to a Data General Eclipse System with Kratos DS-55 software was calibrated for selected ion monitoring (SIM) with perfluorokerosene (PFK) in the EI$^+$ mode over the range of 119–130 amu's by varying the electrostatic accelerating voltage. The capillaries were placed in the temperature-controlled direct probe and introduced into the evacuated source chamber (but not into the source itself) before the scans began accumulating. About 10 s after SIM scans began the samples were introduced into the source at 30 °C and heated at 10 °C/min until the total ion current (TIC) was between 20000–30000 units; this usually occurred when the direct probe monitor registered a temperature from 40 to 60 °C. A typical TIC is shown in Figure 3. The scans were accumulated until the sample was exhausted or the temperature rose above 70 °C. Higher temperatures increased the likelihood of bleedover from previous runs, which may have contaminated the ionizing source or the direct probe. All five fractions of the same nicotinamide sample were run sequentially, with at least an hour of baking the source and the direct probe at 200 °C between different nicotinamide samples. The M$^+$ and the (M + 1)$^+$ peaks for [^{2}H/^{1}H]nicotinamide at 123.0540 and 122.0480 were each scanned with the 122.048 peak used as a lock signal in each scan cycle to correct for magnet drift. Each mass peak was swept >1000 ppm over 68 ms with a 15-ms settling time for a total cycle time of 0.20 s. This sweep width was sufficient to include over 98% of the peak at low resolution. A total of 1000–5000 scans per sample were acquired depending on the size of the sample. It was important to keep the TIC as constant as possible over the entire course of the data acquisitions, as sharp changes in the TIC altered M + 1/M ratios. Reagent grade niacinamide (Sigma) was analyzed by identical mass spectral measurements for the natural abundance M/M + 1 ratio as a check on the accuracy and precision of the method. Ratios were analyzed at various intervals of a single run to check for internal consistency over the course of the scan times. A standard sample of [^{2}H/^{1}H]nicotinamide was run just prior to and immediately after the experimental samples were run. The ^{2}H/^{1}H ratios of these standard runs did not differ by more than 0.05% over the course of the entire set, but day to day differences were larger, necessitating the measurements in each experiment to be taken in one sitting.

Ratios of ^{2}H/^{1}H were calculated with the AREA program from Kratos DS-55. Segments containing 500–2000 scans in which the TIC varied less than 2% were used. The M + 1 peak was corrected for natural abundance ^{13}C by subtracting 7.6% of the intensity of the M$^+$ peak.[2] $^DK_{eq}$ for association of [4-^{2}H]NAD$^+$ with LDH was calculated with the equations derived for the calculation of $^TK_{eq}$ by substituting the M$^+$/M + 1 ratios from the free and bound sides of the dialysis cell for R_F and R_M, respectively.

RESULTS

Isotope Effect Measurements. The isotope effects on the LDH–NAD$^+$ association constant obtained with both [4-^{2}H]NAD$^+$ and [4-^{3}H]NAD$^+$ are given in Table I. The values greater than 1.00 indicate that the bond to the C-4 hydrogen has a smaller zero point energy when NAD$^+$ is bound to the enzyme; i.e., at least one of the vibrational force constants has been decreased by binding to the enzyme (Hartshorn & Shiner, 1972). The average measured ^TK_A of 1.085 can be used to calculate a ^DK_A of 1.060 (Swain et al., 1958). This value is within experimental error of the ^DK_A measured by the isotope ratio technique.

[2] The value of 7.60% comes from experimental observation of the natural abundance M + 1 peak in a standard nicotinamide sample using the SIM protocol described. The value calculated from the natural abundance of ^{13}C, ^{2}H, and ^{15}N is 7.38%.

Table I: Determination of the Isotope Effect on Association by Isotope Ratio Measurements

expt	isotope[a]	R_F[b]	R_M	f_B	^IK_A
1	^2H	0.912	0.971	0.5	1.13 ± 0.03[c]
2	^2H	1.002	1.042	0.5	1.08 ± 0.03
3	^2H	1.008	1.050	0.5	1.08 ± 0.03
4	^3H	0.0926	0.0966	0.5	1.086 ± 0.018[d]
5	^3H	1.876	1.961	0.48	1.094 ± 0.015
6	^3H	0.131	0.137	0.50	1.092 ± 0.015
7	^3H	0.0629	0.0652	0.54	1.068 ± 0.015
8	^3H	0.896	0.937	0.55	1.083 ± 0.015

[a] Measurements of $^1H/^2H$ done by mass spectrometer; measurements of $^1H/^3H$ done with scintillation counting as described under Materials and Methods. [b] R_F, R_M, f_B, and ^IK_A as defined under Materials and Methods and Results. [c] Standard error as calculated directly from error in measurements of the sample. This does not reflect the possibility of contaminating radioactive products, and thus actual errors in ^IK_A may be larger (see Materials and Methods).

Error Analysis. By assuming that the measurements of R_M, R_F, $[NAD]_{free}$, and $[NAD]_{bound}$ are independent, i.e., their covariances are zero, a variance analysis of eq 1 and 2 yields

$$\text{var } {}^IK_A = (R_B/R_F^2)^2 \text{ var } R_F + (1/f_B^2R_F^2) \times [\text{var } R_M + (f_B - 1)^2 \text{ var } R_F + (R_F - R_M)^2 \text{ var } f_B] \quad (3)$$

where $f_B = [NAD]_{bound}/([NAD]_{bound} + [NAD]_{free})$. There are two important features of eq 3: the variance of the isotope effect increases significantly with f_B less than 0.5, and the variance in f_B contributes negligibly to the overall experimental error when R_F and R_M differ by less than 10%. This indicates that the major source of error is in the precision and reproducibility of the measurement of isotopic ratios.

In the mass spectrometric method the standard error of the mean isotope ratio with over 1000 separate measurements decreased to less than 0.2%. The standard deviation of the five separate determinations for each homogeneous solution was closer to 1%, indicating that sample to sample variation in instrumental conditions or sample preparation was the largest source of experimental error. The standard deviation of the two individual ratios is in line with the observed standard deviation of ±0.03 for the calculated isotope effect. Since the final isotope effect calculation is a ratio of ratios, any systematic error that results in a proportional absolute error to the measurement to R_F and R_M will not contribute any systematic error to the calculated isotope effect. Several known and potential sources of proportional systematic errors are known. Instrumental bias in mass spectrometric isotope ratio measurements is common (Beynon, 1960) and was detected on our instrument by using *p*-bromoacetanilide to determine a $^{79}Br/^{81}Br$ ratio (Wan & Anderson, 1987). Small isotope fractionations in the workup resulting from nonquantitative hydrolysis or the detected slow decomposition of nicotinamide under the hydrolysis conditions could also contribute such an error, but should cancel out in the final isotope effect calculation. Similar isotope ratio measurements on nicotinamide derived from NAD^+ have been performed by Kurz and Frieden (1980) using a quadrupole GC–MS. They too experienced difficulties if care was not taken to completely purge the instrument between different samples. Some of the sample to sample variation encountered in the mass spectroscopic measurements could potentially be decreased by the inclusion of an internal isotope standard ratio. Such internal ratios are routinely used in the determination of $^{206}Pb/^{207}Pb$ ratios to ±0.001% by using the $^{204}Pb/^{206}Pb$ ratio as an internal standard (Gulson et al., 1984).

The $^{14}C/^3H$ counting experiments presented different potential sources of error. When identical counting conditions are used and the ^{14}C spillover into the ^3H channel is limited to less than 10% of the total ^3H channel counts, the potential error in the ^3H correction becomes negligible. Any possible error introduced by this correction is further limited because it would affect both R_F and R_M proportionately. With sufficient radioactivity and long enough counting times or repetitions, the standard deviation of the $^{14}C/^3H$ ratio can be decreased to less than 1%. The major source of error lies in impure radioactive material so that the ratios measured are not accurate representations of the concentrations of the free and bound NAD^+. The hydrolytic formation of [U-^{14}C]-ADP-ribose, which binds to LDH (Hinz et al., 1978), and [4-^{3}H]nicotinamide, which does not, resulted in large calculated isotope effects that increased with incubation time in preliminary experiments. This problem was overcome by HPLC purification of the NAD^+ before and after the equilibrium dialysis. Figure 1 shows that the radioactive hydrolytic fragments are well resolved from the NAD^+ but that care must be taken to exclude the trailing [4-^{3}H]nicotinamide. Both NAD^+ assays done as a function of time and the reproduction of the isotope effect by initially adding the NAD^+ to either side of the dialysis membrane indicate that sufficient time was allowed for equilibrium to be obtained. In future experiments the difference in radioactive hydrolytic products may be avoided by using ^3H as a remote label for ^2H in the same portion of the molecule as the ^{14}C, i.e., [4-^{2}H]nicotinamide [8-^{3}H]adenine dinucleotide. These experiments would be analogous to those done to measure the isotope effect on the binding of [1-^{2}H]leucinal to leucine aminopeptidase (Andersson et al., 1985).

Discussion

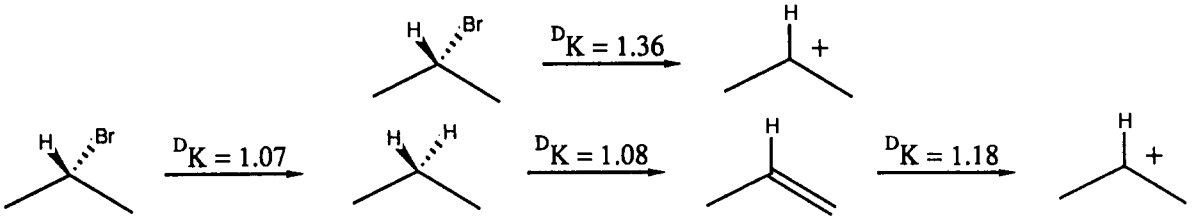
The significant isotope effect on binding of [4-^{2}H]NAD^+ to LDH indicates that one of the vibrational frequencies associated with the C_4–H bond has been decreased. If this is attributed to a single stretching frequency, and we assume that the stretching frequency of NAD^+ in solution is similar to an alkene (Cook et al., 1980, 1981), then by using eq 4 (Lowry & Richardson, 1981) we can calculate that the stretching frequency has decreased ca. 100 cm^{-1} on binding to the enzyme.

$$\nu_F - \nu_B = 1600 \ln {}^DK_A \quad (4)$$

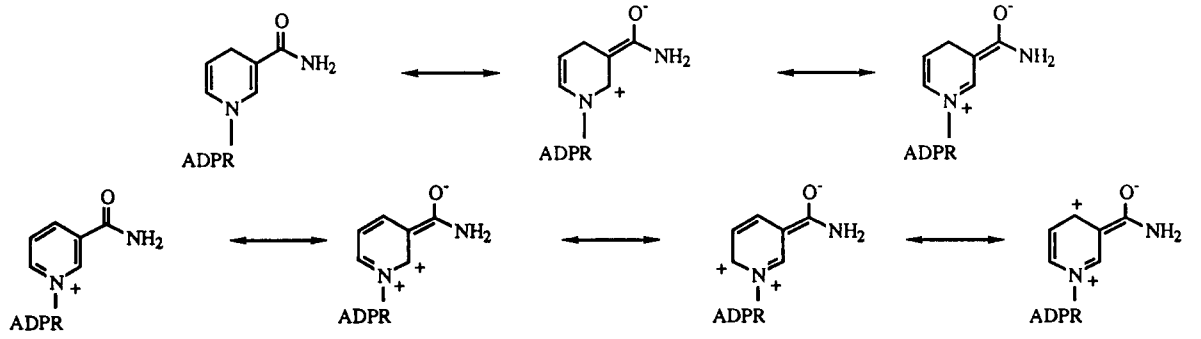
This decrease in frequency could potentially be due to an increase in positive charge at C-4 of the nicotinamide. A reasonable model for comparison is the C–H stretching frequency of aldehydes in which the sp^2 carbon is more electropositive than in the analogous sp^2 alkene. The C–H stretch of aliphatic aldehydes is between 2800 and 2850 cm^{-1}; both acetaldehyde and propenal are 2830 cm^{-1}, while the Ch-2 hydrogen stretch of terminal alkenes is in the 3010–3040-cm^{-1} region (Grasselli & Ritchie, 1975). This difference of ca. 200 cm^{-1} indicates that an increase in positive charge at carbon could decrease the stretching frequency enought to induce a partition factor of over 1.1 and is consistent with a model that specific interactions of the NAD^+ with LDH increase the localized positive charge at Ch-4 and result in the observed isotope effect on binding.

Estimates of the fractionation factor for carbocations have centered on the results obtained from the S_N1 solvolyses of phenylethyl halides (Shiner et al., 1968), which found solvent- and substituent-independent α-deuterium kinetic isotope effects of 1.15 for chlorides and 1.12 for bromides. The bulk of this fractionation factor could be accounted for by the change from sp^3 to sp^2 hybridization (Hartshorn & Shiner, 1972). Recently, McLennan (1987) has argued that the fractionation factors for carbocations based on the phenylethyl halide solvolyses

Scheme I



Scheme II



systematically underestimate the true fractionation factor for carbocations. On the assumption that the carbon–hydrogen bond-stretching force constant is a function of the charge on the carbon, as suggested by an ab initio LCAO calculation of bond lengths of the 2-propyl cation, a "maximum" α-secondary deuterium kinetic isotope effect on solvolysis of isopropyl bromide relative to hydrogen of 1.36 was calculated. With the fractionation factors of the Hartshorn and Shiner (1972), the $^{D}K_{eq}$ for the conversion of the substrate sp^3 bromide to an sp^3 hydrocarbon (1.07) and for the conversion of the sp^3 hydrocarbon to an alkene (1.08), a factor of 1.18 is estimated for the conversion of the alkene to an sp^2-hybridized carbocation as illustrated in Scheme I. This estimated value of 1.18 only indicates that the induction of a positive charge at an sp^2 carbon will result in a detectable change in the fractionation factor of the sp^2 C–H bond that is consistent with the magnitude of the effect measured.

Generalized changes in solvation of the pyridine ring cannot account for the observed effect. Isotope effects on vapor pressure (Van Hook, 1969) and on partitioning into hydrophobic chromatographic phases (Tanaka & Thornton, 1975) are less than 1% per ^2H. Larger effects of 3% and 1–2% have been observed on the transfer of [1-^{2}H]-*N*-methylformamide (Wolfenden & Kirkman, 1983) and β-deuterated carbonyl compounds (Kovach & Quinn, 1983) from water to organic phases, respectively. These changes were attributed to solvation of the carbonyl carbon by H_2O, which increased its sp^3 character. This makes the formamide C–H bond stiffer in H_2O directly and the β C–H bonds stiffer in H_2O by loss of hyperconjugation. A similar decrease in solvation of the nicotinamide carbonyl on binding to LDH would enhance its ability to withdraw electrons from the pyridine ring and lead to the less stiff C–H bond at C-4 that we have observed.

An alternative explanation of the observed isotope effect on association could arise from a less stiff out-of-plane bending mode. In changing from sp^3 to sp^2 hybridization, the sp^2 bond is less stiff because the out-of-plane bending vibration has decreased in frequency significantly more than the stretching freequency increased (Hartshorn & Shiner, 1972). The solvation of the nicotinamide, or the association of the nicotinamide ring with the adenine ring in NAD^+ (Oppenheimer, 1982), may make the out-of-plane vibration stiffer when the NAD^+ is free in solution.

The 10^5 increase in the equilibrium constant for the oxidation of lactate by NAD^+ is consistent with the observed isotope effect, although this involves interactions in the ternary catalytic complex and not the binary complex we are observing. The importance of the 3-carboxamide arm in this interaction was suggested by the demonstration that the stereospecificity of hydride transfer catalyzed by LDH is in excess of 1 in 10^8 (Anderson & LaReau, 1988). This indicated a difference of over 8 kcal/mol of interaction energy in the syn and anti conformations. The induction of positive charge at C-4 in the binary complex is supported by the observation that cyanide adds to NAD^+ in the binary complex with LDH 200–300 times faster than it does free in solution, and the stability constant for adduct formation is increased 400-fold (Gerlach et al., 1965; Burgner & Ray, 1984). If the primary interaction that leads to the charge induction is a polarization of the carboxamide carbonyl, it explains why an isotope effect is observed on the binding of [4-^{2}H]NAD^+ but not [4-^{2}H]-NADH (deJuan & Taylor, 1976). Scheme II shows how polarization of the carbonyl and consequent stabilization of the zwitterionic resonance structures will remove electron density from C-4 of NAD^+ but not NADH. For NAD^+ the resonance structures with the positive charge located at either C-2 or C-6 will be less stable than the structure with the charge at C-4 because of the juxtaposition of two positive charges. Polarization of the carbonyl is not the only potential mechanism for inducing a positive charge at C-4. Cook et al. (1981) have suggested that deforming the aromatic NAD^+ ring could also lead to increased carbocation character at C-4. These isotope effects on the binding of NAD^+ and NADH to LDH demonstrate a corollary derived from the principle that binding energy is used to stress substrates bound at enzyme active sites (Jencks, 1975; Fersht, 1987) and that catalytically efficient enzymes will have internal equilibrium constants close to 1 (Albery & Knowles, 1976): For a substrate–product pair, the one bound less tightly will be more highly stressed and have the largest isotope effect on binding if the strained bond is isotopically labeled.

Extension of the Method. Although in this application the radioactive assays gave more reproducible results, the ability of isotope ratio mass spectrometry to detect isotope effects on binding has been demonstrated. In other systems where no product workup is necessary or where the appropriate ^{14}C and

^3H labels are not practically available, whole atom isotope ratio mass spectroscopy is a practical tool. NAD^+ could be isolated and the glycosidic bond cleaved pyrolytically in the mass spectrometer's direct insertion probe (Ehmke et al., 1980). If the isotopic label can be quantitatively converted to CO_2 or N_2, then the extreme precision of gaseous isotope ratio mass spectrometry (O'Leary, 1980) would be available for the measurement of isotope effects on binding using the natural abundance of ^{13}C and ^{15}N.

Registry No. LDH, 9001-60-9; NAD^+, 53-84-9; [4-^{3}H]NAD^+, 2980-50-9; [4-^{2}H]NAD^+, 60797-91-3; $^2H_2$, 7782-39-0; $^3H_2$, 10028-17-8; D-[1-^{3}H]glucose, 33417-97-9; D-[1-^{2}H]glucose, 10390-17-7; hexokinase, 9001-51-8.

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