Reduced Nicotinamide 8-(Alkylamino)adenine Dinucleotides: Enzyme–Coenzyme Interactions with Different Adenyl Glycosyl Bond Conformations†

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ABSTRACT: Enzyme binding studies have been conducted on several reduced nicotinamide adenine dinucleotide analogues having different substitutions at the 8 position of the adenine. The following analogues were synthesized for this study: 8-bromo-, 8-(methylamino)-, 8-(dimethylamino)-, and 8-(ethylamino)-substituted NADH. The conformation of these analogues was also studied. ^1H and ^{13}C nuclear magnetic resonance analysis showed that there was rotation about the adenine glycosyl bond and that the rotational preference depended on the C8 substituent. The bromo and dimethylamino analogues were predominantly in the syn conformation, while the anti conformation prevailed in the other derivatives as it does in the native NADH. Use of these analogues as coenzymes by *Pseudomonas aeruginosa* transhydrogenase, *Beneckea harveyi* FMN:NADH oxidoreductase, rabbit muscle lactate dehydrogenase, beef heart lactate dehydrogenase, horse liver alcohol dehydrogenase, and yeast alcohol dehydrogenase resulted in enzyme activity in all cases. The bromo and dimethylamino analogues were bound significantly tighter than the other analogues for at least two of the enzymes studied. The data are discussed with respect to the ability of these enzymes to bind nucleotides which are in the syn conformation.

Beillmann & Samama (1974) have suggested that dogfish lactate dehydrogenase and horse liver alcohol dehydrogenase require the nicotinamide ring of nicotinamide adenine dinucleotide to be in the anti conformation about the nicotinamide glycosyl bond for enzymatic activity. This conclusion was based on the observation that 6-methylnicotinamide adenine dinucleotide, a derivative in the nicotinamide ring in which steric hindrance causes a predominantly syn conformation, was not active as a coenzyme nor as a competitive inhibitor. Dinucleotides with anti conformation nicotinamide rings were active or were competitive inhibitors of the two enzymes.

A report from this laboratory (Evans & Kaplan, 1976) examined the specificity of chicken muscle lactate dehydrogenase for the conformation of the adenine ring of NAD^+ by using derivatives of adenosine 5′-monophosphate as competitive inhibitors. The compounds 8-(methylamino)- and 8-aminoadenosine 5′-monophosphate were synthesized and determined to be predominantly in the anti conformation about the adenine glycosyl bond. 8-(Dimethylamino)adenosine 5′-monophosphate was synthesized and determined to be predominantly in the syn conformation. Workers from several laboratories (Evans & Kaplan, 1976; Emerson et al., 1967; Danyluk & Hruska, 1968; Chan & Nelson, 1969; Ikehara et al., 1972; Evans & Sarma, 1974) have shown that adenosine 5′-monophosphate is in the anti conformation. When the analogues were examined as inhibitors for NAD^+ with chicken muscle lactate dehydrogenase, little difference was found in the K_i values of the syn and anti compounds. This was somewhat surprising since, according to crystallographic studies (Abdallah et al., 1975), even syn compounds (8-bromoadenosine diphosphoribose and oxidized nicotinamide 8-bromoadenine dinucleotide) that were predominantly syn in free solution were bound to liver alcohol dehydrogenase as the anti rotamers.

We have extended our previous studies by synthesizing derivatives of reduced nicotinamide adenine dinucleotide: reduced nicotinamide 8-bromoadenine dinucleotide [N(BrA)DH],[1] reduced nicotinamide 8-(dimethylamino)adenine dinucleotide [N[$(CH_3)_2$N-A]DH], reduced nicotinamide 8-(methylamino)adenine dinucleotide [N(CH_3NH-A)DH], and reduced nicotinamide 8-(ethylamino)adenine dinucleotide [N(CH_3CH_2NH-A)DH] (Figure 1). ^{13}C and ^1H NMR have verified that the conformation about the glycosyl bond of the adenine ring in these dinucleotides is conserved from the mononucleotide: the two former derivatives have a predominantly syn conformation and the two latter compounds are predominantly anti.

Using these compounds as coenzymes, we have performed binding and kinetic studies with pyridine nucleotide dependent enzymes. Some enzymes, notably lactate dehydrogenases, appear to have a preference for coenzyme derivatives with adenine rings possessing an adenine glycosyl bond in the syn conformation. We interpret these data to mean that at least these enzymes can bind the coenzyme when the adenine ring is in the syn conformation.

Experimental Procedures

The synthesis of the coenzyme analogues was based on the method of Lee et al. (1974). Substantial modifications were made. All alkylamino analogues were synthesized from N(BrA)DH.

Synthesis of N(BrA)D$^+$. In 125 mL of 1 M sodium acetate, pH 4.8, 5 g of NAD^+ was dissolved and the pH was adjusted to 4.5. A total of 1.5 mL of liquid bromine was added in 0.3-mL aliquots at ~2-min intervals or until full solubility of

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[1] Abbreviations used: BrAMP, 8-bromoadenosine 5′-monophosphate; $(CH_3)_2$N-AMP, 8-(dimethylamino)adenosine 5′-monophosphate; CH_3NH-AMP, 8-(methylamino)adenosine 5′-monophosphate; N(BrA)D(H), (reduced) nicotinamide 8-bromoadenine dinucleotide; N[$(CH_3)_2$N-A]DH, reduced nicotinamide 8-(dimethylamino)adenine dinucleotide; N(CH_3NH-A)DH, reduced nicotinamide 8-(methylamino)adenine dinucleotide; N(CH_3CH_2NH-A)DH, reduced nicotinamide 8-(ethylamino)adenine dinucleotide.

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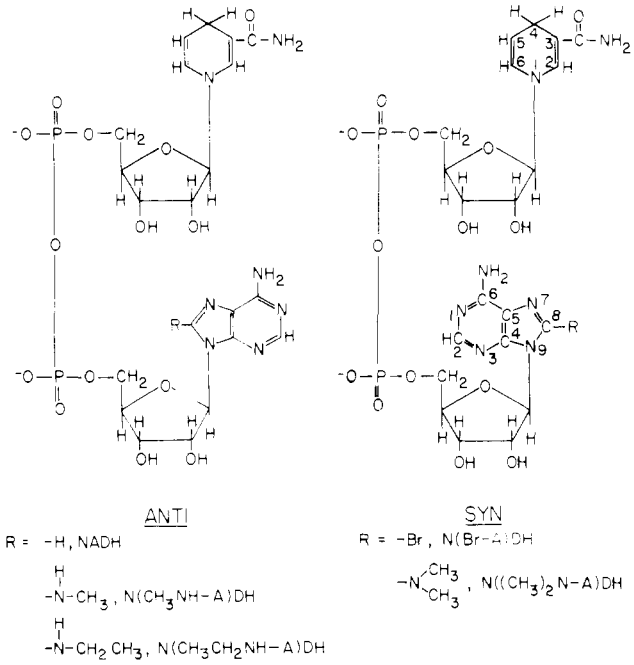

FIGURE 1: Reduced nicotinamide adenine derivatives comparing syn and anti conformations.

the bromine. The pH was allowed to drop as the reaction proceeded as first directed for the synthesis of BrAMP (Ikehara et al., 1969).

Fifteen minutes after the last addition, the solution was extracted 5 times with equal volumes of chloroform or carbon tetrachloride, the pH was adjusted to 1–2 with nitric acid, and the coenzyme was precipitated with 5 volumes of −20 °C acetone. The material was allowed to precipitate at −20 °C for at least 2 h and then centrifuged at 6000*g* for 20 min. The precipitate was dissolved in 1 L of water and the pH was adjusted to 4.5. This solution was applied to a DEAE-Sephadex A-25 column (formate form; 4.5 × 35 cm), and the column was eluted with a 0–0.15 M formic acid gradient (2 L/side). Fractions yielding a 327-nm absorbing adduct with cyanide (Colowick et al., 1951) were pooled and lyophilized. The resulting white powder was shown by ^{1}H NMR to contain neither a detectable adenine 8 proton resonance nor any nucleotide contaminants (Evans & Sarma, 1974). Yields were routinely 33–38%.

Synthesis of N(BrA)DH. In 250 mL of 0.1 M Tris containing 6% ethanol, N(BrA)D$^+$ (4.1 g) was dissolved and the pH was adjusted to 9.5. Enough yeast alcohol dehydrogenase was added to cause complete reduction within 2 h. The A_{265}/A_{340} ratio was monitored, and the pH was maintained at 9.5 by periodic additions of 1 N NaOH. When the ratio reached an end point (usually 2.9), the solution was diluted to 2 L and applied to a QAE-Sephadex A-25 column (chloride form; pH 9–10; 4.2 × 35 cm). The column was washed with a linear gradient of 0–0.3 M LiCl (2 L/side) with the pH adjusted to 9–11 with LiOH. The column was then washed with 0.3 M LiCl (pH adjusted to 9–11) until all N(BrA)DH was eluted. Fractions with an A_{265}/A_{340} ratio less than 2.9 were pooled and precipitated with 10 volumes of −20 °C 2-propanol. The precipitate was redissolved in a minimum volume of water and the precipitation was repeated twice. The precipitate was redissolved in water. The pH was adjusted to 9–10 with LiOH, and the solution was lyophilized. The yields were routinely 65–75%.

Synthesis of N(alkylamino-A)DH. The procedures for synthesis of N(CH$_3$NH-A)DH, N(CH$_3$CH$_2$NH-A)DH, and N[(CH$_3$)$_2$N-A]DH were similar; the only exception was that liquid ethylamine was dissolved in water and the pH was adjusted to 11.0 with HCl, while the solid dimethylamine hydrochloride and the solid methylamine hydrochloride were dissolved in water and the pH was adjusted to 11 with NaOH. One gram of N(BrA)DH was dissolved in H_2O and added to a 100-mL solution of the appropriate amine at pH 11 and in a 200-fold molar excess. This material was heated in a sealed container to 40 °C and examined for a bathochromic shift after 16 h. At 20 h, if no further bathochromic shift occurred, the mixture was rotary evaporated, redissolved in a small volume of water, precipitated with at least 10 volumes of −20 °C 2-propanol, and stored for 12 h. This precipitate was collected by centrifugation and dissolved in 500 mL of water. The pH was adjusted to 10 and applied to a QAE-Sephadex A-25 column (4.2 × 35 cm). The alkylamino derivative was eluted with a 0–0.3 M LiCl (4 L total) gradient and then sufficient 0.3 M LiCl to complete elution. The material was rotary evaporated and precipitated with 2-propanol and dried in a manner similar to that of N(BrA)DH. The yield of this step was 70%. The overall yield starting from NAD$^+$ was 16–20%. The structure of the product was confirmed from the ^{1}H and ^{13}C NMR spectra.

Enzymatic Analysis. Pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa* was a kind gift from Laura Morriss of this laboratory. Kinetic measurements were made according to the method of Cohen & Kaplan (1970) in the presence of 5 × 10^{-4} M 2′AMP.

Fluorescent binding assays of beef heart lactate dehydrogenase, rabbit muscle lactate dehydrogenase, and horse liver alcohol dehydrogenase were conducted according to the method of McKay & Kaplan (1964).

Kinetic measurements of yeast alcohol dehydrogenase were made according to the method of Wills (1976) at an acetaldehyde concentration of 3.4 × 10^{-2} M.

Kinetic measurement of FMN:NADH oxidoreductase from *Beneckea harveyi* were made with the coupled system according to the method of Jablonski & DeLuca (1978).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra were recorded on a Varian HR 220 spectrometer. ^{13}C NMR spectra were recorded on a Bruker WH 270 spectrometer. All data were collected in the Fourier transform mode by using either a Nicolet 1080 or an Aspect 2000 computer. All samples were dissolved in deuterium oxide. ^{1}H chemical shifts were reported in parts per million from internal tetramethylsilyl propionate, with downfield shifts positive. Data acquisition parameters for the ^{1}H spectra were as follows: 8192 data points; 2.500-Hz sweep width; 60° flip angle; 2-s relaxation delay; number of scans was typically 32. Data acquisition parameters for the ^{13}C spectra were as follows: 32 768 data points; 12 195-Hz sweep width; 80° flip angle; 7-s relaxation delay with broad-band proton radiation only during the relaxation delay time; number of scans ranged from 5000 to 10 000.

Results and Discussion

Ultraviolet Absorbance Characteristics. Wavelength maxima and minima, molar extinction coefficients, and ascending paper chromatography data are listed in Table I. All reduced derivatives show an increase in the adenine molar extinction coefficients at the peak absorbance. This ranges from an 8% increase in the case of N(BrA)DH to a 28% increase in the case of N(CH$_3$NH-A)DH. There is very little effect by the dihydronicotinamide absorbance on 264-nm absorbance, the absorbance maximum of N(BrA)DH. There is a contribution by the shoulder of the dihydronicotinamide

Table I: Spectroscopic and Ascending Paper and Chromatography

compd	λ_{max} (nm)	ϵ_M (L mol^{-1} cm^{-1})	λ_{min} (nm)	ϵ_M (L mol^{-1} cm^{-1})	R_{st}[b]
NADH	259 (15)	14400	232	6900	1.00
N(BrA)DH	264[a]	15600	237	6550	1.09
N[$(CH_3)_2$N-A]DH	275[a]	16700	242	4100	1.41
N(CH_3NH-A)DH	279[a]	18500	241	4300	0.98
N(CH_3CH_2NH-A)DH	279[a]	17400	241	3500	1.21

[a] Recorded at pH 8.5. [b] Solvent was ethanol–1 M ammonium acetate, pH 7.5 (2:1) on Whatman No. 1 paper. R_f for NADH was 0.273. R_{st} are R_f values standardized to the R_f of NADH.

absorbance to the extinction coefficients of N(alkylamino-A)DH adenine at their absorbance maxima of 275–279 nm. The modifications of the adenine ring may also contribute to increased extinction coefficients. Other workers have found this to be the case for modifications of the adenine ring of mononucleotides (Holmes & Robins, 1964).

The bathochromic shift of the adenine absorbance maximum is the result of the entire peak shifting rather than a broadening effect as seen by examination of the minima around 240 nm (Table I). These minima also show a bathochromic shift, and, as the peaks move away from the absorbance of general electronic transitions below 220 nm, the molar extinction coefficients of the minima decrease due to less overlap with this strongly absorbing area.

The extinction coefficient at 340 nm of the 1,4-dihydronicotinamide peak is unchanged as judged by the lack of a detectable change in the A_{340}/A_{355} ratio of the derivatives compared to that of NADH and by the examination of the absorbance spectrum of the model compound 8-[(6-aminohexyl)amino]-5′-AMP.

The previous method (Lee et al., 1974) for synthesis of 8-(alkylamino)adenyl dinucleotide derivatives from the brominated derivative was carried out in dimethyl sulfoxide. It was assumed that at elevated temperatures oxidation of the reduced nicotinamide would occur in aqueous solutions. This would result in loss of product, since the oxidized coenzyme would be degraded by base attack on the pyridine glycosyl bond. However, in aqueous solution at pH 10 or 11, N-(BrA)DH is quite stable, even at 40 °C for 24 h. We estimate 10% of the nucleotide is oxidized and degraded under these conditions. Due to the convenience of aqueous solutions, we now recommend running the reaction in water. It is also advantageous to have the alkylamine replacement go to completion because the complete separation of N(alkylamino-A)DH from N(BrA)DH is difficult with the method used.

Nuclear Magnetic Resonance Spectra Properties. The 220-MHz ^{1}H nuclear magnetic resonance spectra of the dinucleotide derivatives are shown in Figure 2. The ribose resonances in general are sensitive to the nature of the 8 substituent. However, the chemical shift of the adenine ribose 2′ proton is most sensitive to the orientation of the adenine ring about the glycosyl bond. For instance, with AMP derivatives, the chemical shift of the adenine 2′ proton shifts of anti nucleotides is 0.5 ppm downfield of that of syn nucleotides (Evans & Kaplan, 1976). The 2′ proton chemical shifts of NADH (4.65 ppm), N(CH_3NH-A)DH (4.70 ppm), and N(CH_3CH_2NH-A)DH (4.74 ppm) are similar (Figure 2). The 2′ proton chemical shifts of N(BrA)DH (5.32 ppm) and N[$(CH_3)_2$N-A]DH (5.34 ppm) are much further downfield (Figure 2). These data, according to evidence presented previously (Evans & Kaplan, 1976), indicate that the adenine ring of N(CH_3NH-A)DH and N(CH_3CH_2NH-A)DH and NADH in solution is predominantly in the anti conformation

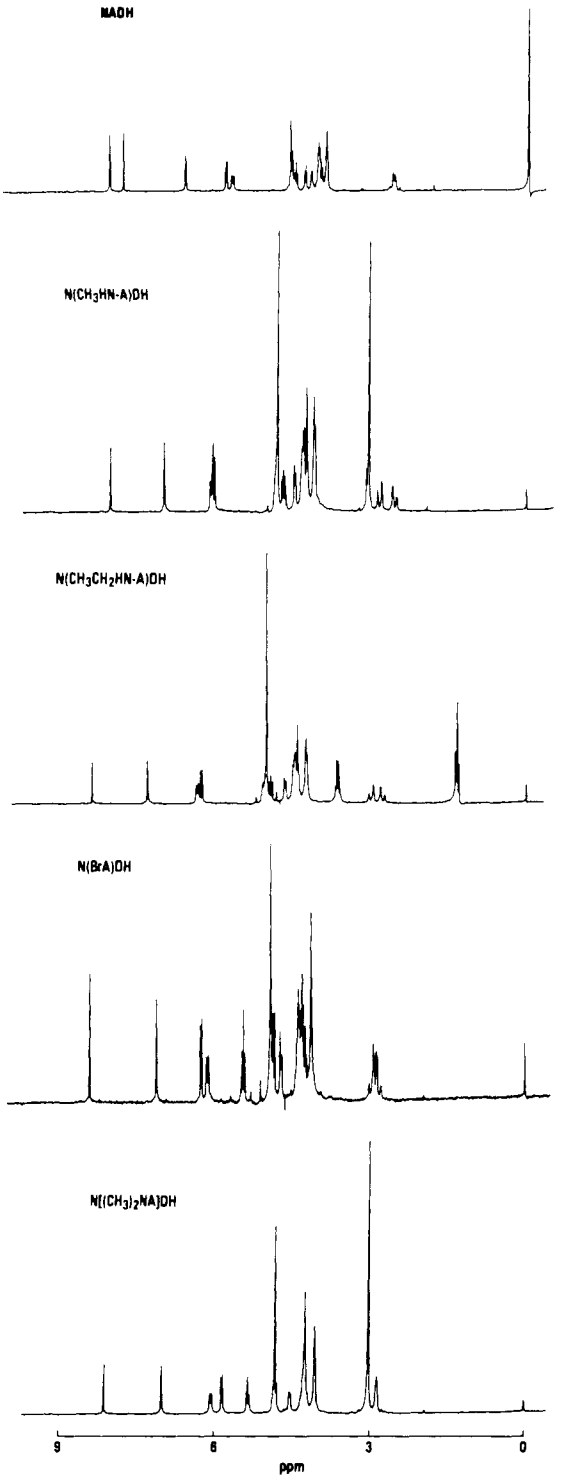

FIGURE 2: ^{1}H NMR of NADH, N(CH_3NH-A)DH, N(CH_3CH_2NH-A)DH, N(BrA)DH, and N[$(CH_3)_2$N-A]DH. See the text for chemical shifts of 2′-adenine ribose resonances.

about the glycosyl bond, while that of N(BrA)DH and N-[$(CH_3)_2$N-A]DH is predominantly in the syn conformation.

To further describe the conformation about the adenine glycosyl bond, we measured the coupling constant $^3J_{C4-H1'}$ from the ^{13}C NMR spectrum for two of the dinucleotides. The results were compared with those of the corresponding adenine mononucleotides (Figure 3). This coupling constant was 5.1 Hz in both N[$(CH_3)_2$N-A]DH and $(CH_3)_2$N-AMP, while the value was 1.8 Hz in both N(CH_3NH-A)DH and CH_3NH-AMP. This directly shows the substantial difference in the torsion angle about the C1′–N5 bond between the mono- and

Table II: Chemical Shifts of Pyridine Ring 4A and 4B Protons

compd	δ_1	δ_2	$\Delta\delta$
$N(CH_3NH\text{-}A)DH$	2.83	2.56	0.27
$N(CH_3CH_2NH\text{-}A)DH$	2.87	2.67	0.20
$N[(CH_3)_2N\text{-}A]DH$	2.85	2.85	*a*
N(BrA)DH	2.90	2.78	0.12

[a] Not detected.

dialkylamino-substituted derivatives. Further, it shows that the conformation in the mononucleotides is conserved in the dinucleotides. The angle, denoted as χ, can be used to describe the conformation about the glycosyl bond. χ is defined as zero when the N9–C8 bond in the purine moiety is cis planar to the O1′–C1′ bond of the ribose moiety. In general, the region between 0 and 70° is the anti region, and the region between 210 and 260° is the syn region. We have previously estimated the glycosyl torsion angle χ for CH_3NH-AMP to be 15° (Evans & Kaplan, 1976). The coupling constant data for the dimethylamino derivatives suggest an average value of χ of ~225°. These values are in fairly good agreement with theoretical calculations of Pohorille et al. (1978). The Karplus-type relationship of the C–C–C–H coupling constant has been previously used to monitor the glycosyl conformation in several nucleosides (Schweizer et al., 1973; Lemieux et al., 1972).

The anti conformation appears to be stabilized by an intramolecular hydrogen bond involving a proton of the exocyclic amine substituted at the C8 position and the ribose 5′-oxygen (Evans & Kaplan, 1976; Pohorille et al., 1978; Evans et al., 1978; Evans & Wright, 1980). However, both experimental and theoretical studies indicated that syn conformers are in equilibrium with the anti conformers. Likewise, in the syn nucleotide $(CH_3)_2N$-AMP, some anti conformation is also present. The preferences for the anti form are not appreciably affected by the N-alkyl chain length (Evans et al., 1978).

Another significant difference exists in the ^1H NMR spectrum of the dinucleotides. The methylene protons 4A and 4B of the 1,4-dihydronicotinamide ring exhibit a chemical shift difference of 0.27 ppm in $N(CH_3NH\text{-}A)DH$ (Table II). The chemical shift difference is only 0.10 ppm in the parent compound NADH. No chemical shift difference is detected when a destacking solvent such as methanol is used. This phenomenon is now being studied in our laboratory.

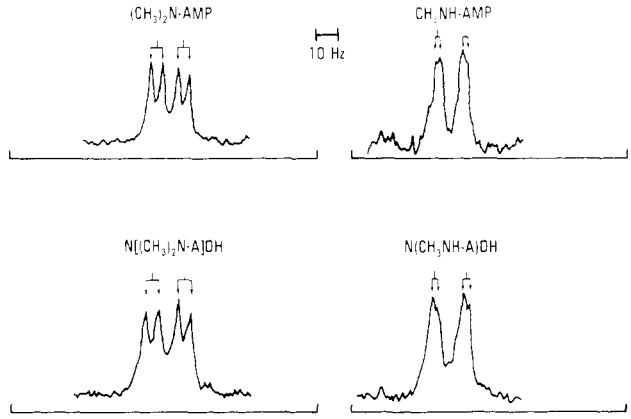

FIGURE 3: ^{13}C NMR resonance of adenine C4 with proton coupling present. The smaller coupling constant $^3J_{C4\text{-}H1'}$ depends on the glycosyl conformation, and the larger coupling constant $^3J_{C4\text{-}H2}$ remains constant. $^3J_{C4\text{-}H1'}$ is ~5.1 Hz in both $N[(CH_3)_2H\text{-}A]DH$ and $(CH_3)_2$-AMP, while it is ~1.8 Hz in both $N(CH_3NH\text{-}A)DH$ and CH_3NH-AMP. The concentration level was 0.1–0.2 M and pD 8.

Enzyme Interactions. Kinetic data from the following six enzymes, *P. aeruginosa* transhydrogenase, *B. harveyi* FMN:NADH oxidoreductase, beef heart lactate dehydrogenase, rabbit muscle lactate dehydrogenase, yeast alcohol dehydrogenase, and horse liver alcohol dehydrogenase, are shown in Table III. Kinetic constants were calculated from linear regression formulas. All coefficients of correlation for linear regression determinations were greater than 0.940. Apparent Michaelis constants and apparent dissociation constants for NADH agreed well with known literature values: *P. aeruginosa* K_m = 0.15 mM (Cohen & Kaplan, 1970), *B. harveyi* FMN:NADH oxidoreductase K_m = 6.0 mM (Jablonski & DeLuca, 1978), beef heart lactate dehydrogenase K_d = 0.8 mM (Cohen & Kaplan, 1970), and horse liver alcohol dehydrogenase K_d = 0.31 μM (Theorell & Winer, 1959). Literature values for rabbit muscle lactate dehydrogenase and yeast alcohol dehydrogenase at the acetaldehyde concentration used were not available. All coenzyme analogues were active with all the enzymes tested.

Yeast alcohol dehydrogenase was the only enzyme that had significantly lower apparent Michaelis constants for analogues whose solution conformations are predominantly anti. Abdallah et al. (1975) also found with this enzyme a predomi-

Table III: Kinetic and Binding Data for Pyridine Nucleotide Dependent Enzymes and Coenzyme Derivatives

	NADH	$N(CH_3NH\text{-}A)DH$	$N(CH_3CH_2NH\text{-}A)DH$	N(BrA)DH	$N[(CH_3)_2N\text{-}A]DH$
		P. aeruginosa Transhydrogenase			
K_m (mM)	0.16	0.92	0.89	0.15	0.79
V_{max}[a]	1.00	1.99	1.76	0.64	2.11
		B. harveyi FMN:NADH Oxidoreductase			
K_m (mM)	6.2	14	5.8	7.0	3.8
V_{max}[a]	1.00	0.70	0.71	0.52	0.17
		Beef Heart Lactate Dehydrogenase			
K_d (μM)	0.95	1.0	1.2	0.50	0.30
V_{max}[a]	1.00	0.51	0.58	0.52	0.29
		Rabbit Muscle Lactate Dehydrogenase			
K_d (μM)	1.1	2.5	1.8	0.26	0.084
V_{max}[a]	1.00	0.78	0.76	0.52	0.40
		Yeast Alcohol Dehydrogenase			
K_m (mM)	2.3	4.6	1.8	11	16
		Horse Liver Alcohol Dehydrogenase			
K_d (μM)	0.31	0.65	0.61	0.36	0.12
V_{max}[a]	1.00	0.64	0.37	0.93	0.43

[a] Values have been normalized to the activities for NADH set at 1.00.

nantly anti conformation coenzyme (NAD^+) that has a lower K_m than a coenzyme with a predominantly syn conformation [N(BrA)D^+]. They also found, by crystallographic means, that N(BrA)D^+ was bound in the active site in the anti conformation.

In the case of the lactate dehydrogenases from beef heart and rabbit muscle, binding of the coenzyme to the enzyme was measured by protein fluorescence. The enzymes bind the coenzymes N(BrA)DH and N[$(CH_3)_2$N-A]DH with lower dissociation constants than those of the other three coenzymes including NADH. The two tighter binding coenzymes are both predominantly in the syn conformation. The data presented here showing an apparent preference for predominantly syn coenzymes suggest that the syn conformation is a proper orientation for binding to these two enzymes and that the coenzymes can be bound in the syn conformation in the active site of the enzyme. Horse liver alcohol dehydrogenase, *P. aeruginosa* transhydrogenase, and *B. harveyi* FMN:NADH oxidoreductase do not appear to discriminate according to the adenine–ribose glycosyl torsion angle. The data for transhydrogenase shows that maximal activity is dependent on the release of the coenzyme. As the Michaelis constant decreases, so does maximal velocity. This agrees with the ping-pong bi bi mechanism postulated by Cohen & Kaplan (1970).

In conclusion, we have synthesized new active NADH analogues which conserve the adenine glycosyl torsion angle of AMP analogues. Two lactate dehydrogenases, beef heart and chicken muscle, appear to prefer NADH analogues with a syn conformation about the adenine glycosyl bond.

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