Pyridine Coenzyme Analogues. Synthesis and Characterization of α- and β-Nicotinamide Arabinoside Adenine Dinucleotides†

Bernard L. Kam,‡ Olaf Malver, Thomas M. Marschner, and Norman J. Oppenheimer*
Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, California 94143
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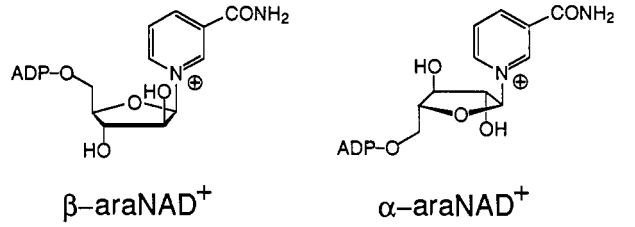
ABSTRACT: The synthesis and characterization of a new pyridine coenzyme analogue containing a nicotinamide arabinonucleotide moiety are reported. The redox potentials are −339 mV for β-oxidized nicotinamide arabinoside adenine dinucleotide and −319 mV for α-oxidized nicotinamide adenine dinucleotide, and the λ_{max} is 346 and 338 nm for β- and α-reduced nicotinamide arabinoside adenine dinucleotides (araNADH), respectively. Anomerization of the reduced analogues leads to a 5:1 ratio of α-araNADH to β-araNADH at 90 °C. These results establish that the relative configuration of the 2′-hydroxyl to the base is the primary determinant for the configuration-dependent changes in λ_{max}, the redox potential of the pyridine nucleotides, and the preferred anomeric configuration of the reduced coenzymes. Comparison of the ^1H and ^{31}P NMR spectral data of the analogues with those for the ribo coenzymes is reported and the conformational analysis discussed. The coenzyme properties of the arabino analogues have been evaluated with yeast and horse liver alcohol dehydrogenases. Both the α- and β-anomers are found to serve as coenzymes, and the stereochemistry of hydride transfer is identical for both anomers.

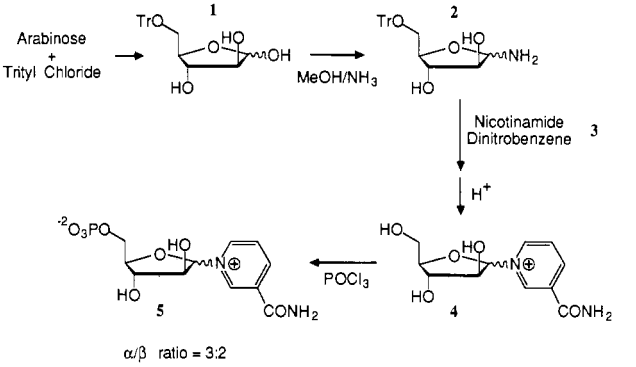
Analogues of pyridine coenzymes have long been important in the study of the steric constraints and mechanism of dehydrogenases, ADP-ribosyltransferases, and other coenzyme-utilizing enzymes. Modifications have focused primarily on the pyridine ring, with special emphasis on the 3-substituent because of their ease of synthesis (Anderson et al., 1959), and to a lesser extent on the adenine moiety [see Anderson (1982) for a comprehensive review]. There have been few investigations directed at synthesis of analogues containing modifications of the nicotinamide sugar moeity (Goebbeler & Woenckhaus, 1966; Woenckhaus et al., 1964; Woenckhaus & Jeck, 1970). The lack of such analogues represents a significant omission since the sugar moiety can influence directly the chemical properties of the coenzyme, can alter coenzyme binding to dehydrogenases, and has been suggested to play an important mechanistic role (Oppenheimer, 1986a).

In this paper we present the synthesis and biochemical characterization of both the α- and β-anomers of a new NAD^+ analogue containing a nicotinamide arabinonucleoside moiety as shown in Structure I. The procedures developed for this synthesis, which are based on the general properties of 5′-*O*-tritylpentofuranosylamine intermediates (Kam & Oppenheimer 1979a,b), represent a generalized approach to the synthesis of a wide range of NAD^+ analogues. The structure–function questions to be addressed focus on the effects that alterations in configuration of the 2′-hydroxyls will have upon (1) the chemical and physical properties of ribo- and arabinonucleotides, (2) the conformation of the sugar moieties, and (3) the binding and utilization of the analogues by pyridine nucleotide dependent enzymes.

Structure I



Scheme I



Experimental Procedures

Materials

Yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, Hepes[1] and Pipes buffers, and semicarbazide hydrochloride were purchased from Sigma. β-NAD^+ and β-NADH were also from Sigma. Acetaldehyde and propanal

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* Address correspondence to this author.
‡ Present address: Institute of Chemistry, University of Ouagadougou, Burkina Faso (West Africa).

[1] Abbreviations: araNMN$^+$, oxidized nicotinamide arabinomononucleotide; araNAD$^+$, oxidized nicotinamide arabinoside adenine dinucleotide; araNADH, reduced nicotinamide arabinoside adenine dinucleotide; riboNADH, reduced nicotinamide riboside adenine dinucleotide; ADH, alcohol dehydrogenase; TBA, tetrabutylammonium; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; DMF, dimethylformamide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TSP, 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography. In the NMR spectra A is used to designate resonances of the adenine moiety and N is used to designate resonances of the nicotinamide moiety.

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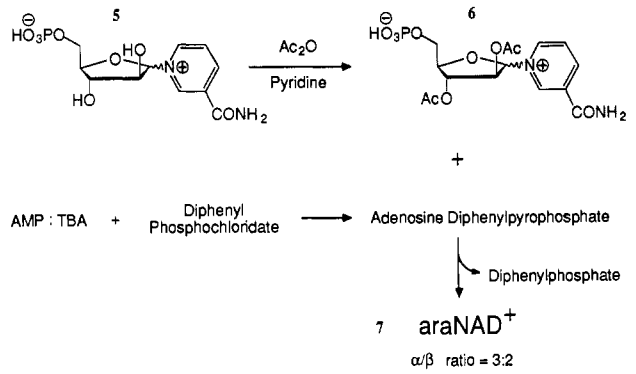
Scheme II



were from Aldrich and were distilled before use. [U-$^2H_5$]-Ethanol was purchased from KOR Isotopes. Solvents were either spectrograde or freshly distilled prior to use. All synthetic reagents were of the highest quality commercially available and used without further purification.

Synthesis

D-(−)-*Nicotinamide Arabinofuranoside* (**4**). The synthesis of the nicotinamide arabinonucleoside was conducted as outlined in Scheme I. Tritylation of D-(−)-arabinose was conducted according to the procedure of Kam and Oppenheimer (1979a) with the following correction; the concentration of arabinose in the reaction mixture should be *0.7 mmol/mL of pyridine*. The crude product was purified by flash chromatography on silica gel. It was eluted first with chloroform to remove triphenylmethanol and then with ethyl acetate to recover the product. The tritylarabinose **1** was obtained as a pale yellow syrup that was dissolved in saturated methanolic ammonia at 0 °C and incubated overnight to give the tritylarabinosylamine **2** (Kam & Oppenheimer, 1979b). The nicotinamide arabinonucleoside was prepared according to the procedure of Kam and Oppenheimer (1979b) for the synthesis of nicotinamide ribonucleoside. Tritylarabinosylamine was added to a methanolic solution of *N*-1-nicotinamide-2,4-dinitrobenzene (3), where it reacts to give the trityl nucleoside.[2] After 4 h ammonia was bubbled through the solution at 0 °C to destroy excess **3**, and the solvent was then removed by rotoevaporation (the bath temperature was maintained below 30 °C). The 5′-trityl group was removed by incubating the residue in 0.1 N HCl for 15 min at room temperature, and the triphenylmethanol was then filtered off. The nicotinamide arabinonucleoside **4** was precipitated with acetone/ether 1:1 by volume and was recovered as a 3:2 mixture of α:β anomers as determined by ^1H NMR.

α,β-Nicotinamide Arabinonucleotide (**5**). The phosphorylation and coupling with AMP were conducted as outlined in Scheme II. The arabinonucleotide was prepared by phosphorylating **4** with $POCl_3/H_2O$ in triethyl phosphate according to the method of Yoshikawa et al. (1967). The reaction mixture was left overnight at 0 °C and then quenched with ice water. Evaporation of the water and precipitation in cold acetone gave a white, hygroscopic precipitate. TLC and ^1H NMR spectroscopy showed quantitative conversion to mononucleotide.

α,β-araNAD$^+$ (**7**). The nicotinamide arabinoside adenine dinucleotide was synthesized via the Michelson (1964) method for coupling of nucleotides to generate a pyrophosphate linkage. Because araNMN$^+$ is insoluble under the reaction conditions, it is necessary to increase the solubility by acetylation of the 2′- and 3′-hydroxyls of the arabino moiety. A solution of α,β-araNMN$^+$ (750 μmol) in 1.5 mL of water was added to a rapidly stirred solution of 15 mL of acetic anhydride in 27 mL of pyridine. The reaction mixture was stirred at room temperature for 2 h and at 5 °C for 10 h. The solvent was removed in vacuo (0.2 mmHg) with a bath temperature below 40 °C. The residue was treated with aqueous pyridine to destroy any mixed anhydride (Saneyoshi, 1971) and after removal of solvent gave a yellow oil that was used directly in the coupling reaction. TLC and ^1H NMR spectroscopy showed complete conversion of araNMN$^+$ (**5**) to the 2′,3′-diacetyl nucleotide **6**.

Although we have accomplished coupling by activating either AMP or araNMN$^+$, only the procedure for araNMN$^+$ activation is presented. Diphenyl phosphochloridate (250 μL) and tri-*n*-butylamine (250 μL) were added under N_2 to a solution of 750 μmol of **6** in 5 mL of freshly distilled dioxane and 3 mL of dry DMF. After the solution was stirred at room temperature for 2 h, the solvent was removed in vacuo with a bath temperature not exceeding 30 °C. Addition of ethyl ether (100 mL) generated an oily precipitate. The mixture was stirred at 0 °C for 30 min, and then the ether phase was decanted and residual ether was removed in vacuo. The tetrabutylammonium (TBA) salt of AMP (1.5 μmol) was dissolved in 5 mL of DMF and then added, along with 5 mL of pyridine, to the yellow oil. The solution was stirred for 10 h at room temperature, and then the solvent was removed in vacuo. Residual solvent was removed by coevaporation with dry methanol (3 × 15 mL). The residue was dissolved in 30 mL of ammonia-saturated methanol and stirred for 4 h at 0 °C to deacetylate the arabinose moiety. After the solvent was stripped off, the residue was dissolved in water (200 mL), adjusted to pH 6.5 with dilute formic acid, and applied to a Dowex AG-1 (formate) column (5 × 25 cm). The column was eluted with a 0–0.5 N formic acid linear gradient, 2 L total. The araNAD$^+$ eluted as a single peak at 0.07 N formic acid with no resolution of anomers. A total of 510 μmol of araNAD$^+$ (**7**) was obtained (yield 67%), and an additional 114 μmol of araNMN$^+$ was recovered. Separation of the anomers was achieved by HPLC on a Whatman Partisil M9 SAX column eluted isocratically with 7 mM ammonium phosphate, pH 3.5. Fractions containing the individual anomers of araNAD$^+$ were pooled and lyophilized. The individual anomers were >99% pure on the basis of analytical HPLC.

α,β-araNADH. Reduction of either α- or β-araNAD$^+$ was conducted enzymatically on a preparative scale (typically 5–10 mg at a time) with yeast alcohol dehydrogenase. Reaction mixtures were incubated at 30 °C and consisted of 50 mM Hepes buffer, pH 8.0, 1 M ethanol, and 10 mM semicarbazide. The extent of reaction was monitored spectrophotometrically, and sufficient enzyme was added to complete the reduction in less than 30 min. The reduced dinucleotides were purified by ion-exchange chromatography on a Pharmacia FPLC system according to the method of Orr and Blanchard (1984) for the purification of β-NADH.

Enzyme Kinetics. The kinetic parameters were measured at 30 °C in stoppered 1-mL cuvettes on a Hitachi 100-80 UV/vis spectrophotometer interfaced to an Apple II computer. The wavelength was set to correspond to the λ_{max} for the particular dinucleotide used in the assay. Stock enzyme solutions were prepared in 50 mM Pipes buffer, pH 7.2. All reactant concentrations were determined enzymatically as

[2] The yield of the Zincke reaction can vary from greater than 70% to as little as 10% depending upon the amount of residual ammonia present, the extent of formation of diribosylamine, and the partitioning between dinitroaniline release and reversal back to nicotinamide dinitrobenzene (Kam & Oppenheimer, 1979b; Walt et al., 1984).

described by Cook et al. (1980). Reactions were conducted in 50 mM Hepes buffer, pH 8.0. Semicarbazide (10 mM) was included in those yeast alcohol dehydrogenase assays where acetaldehyde was being formed. The stock semicarbazide solution was adjusted to pH 8 with KOH before use. The enzyme concentration used in the assay was adjusted to give rates of 0.01–0.1 OD/min at the λ_{max} of the reduced coenzyme. Initial rates were measured by linear regression. Kinetic parameters were measured by nonlinear regression using the programs of Cleland (1979). Ethanol and acetaldehyde were used as substrates for yeast alcohol dehydrogenase at saturating concentrations of 75 and 250 mM, respectively. For studies with horse liver alcohol dehydrogenase (oxidation of reduced dinucleotide), saturating concentrations of propanal could not be reached without substantial substrate inhibition. The concentrations of propanal as well as the dinucleotide were therefore varied, and the observed kinetic parameters were extrapolated up to saturating levels of both dinucleotide and aldehyde (Cleland, 1977).

NMR Spectroscopy. Proton nuclear magnetic resonance spectra were acquired at 500 MHz on a General Electric GN-500 instrument interfaced to a Nicolet 1280 computer and at 360 MHz on a Bruker HXS-360 NMR spectrometer equipped with a Nicolet Technologies 180 computer/Fourier transform system. Samples at 500 MHz were 1 mM in coenzyme and were prepared in 25 mM phosphate buffer, pD 7.8, with 50 μM EGTA to suppress paramagnetic broadening. Samples were lyophilized twice from 99.8% D_2O and then dissolved in 100.0% D_2O. The probe temperature was maintained at 20 °C, unless otherwise noted, and 5-mm NMR tubes were used. Typically, 256 scans were acquired with a spectral width of 6000 Hz, using 16K data points, for 500-MHz spectra and 3610 Hz, using 16K data points, for 360-MHz spectra. The pulse width was set to correspond to a 45° tip angle, and a 0.5-s post acquisition delay was used to allow for relaxation. For measurement of the sugar coupling constants, the free induction decay was apodized with a double exponential to enhance resolution. Chemical shifts were meausred relative to internal 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionic acid (TSP). Partially relaxed spectra were obtained by using a $(180°-\tau-90°-\text{acquisition})_n$ pulse sequence setting in order to null the desired resonances (methines or methylenes).

^{31}P NMR spectra were acquired at 97.3 MHz on a home-built spectrometer interfaced to a Nicolet 1180 computer. The concentration of the analogue was 1 mM and was prepared in 25 mM Hepes buffer, pH 7.8, containing 50 μM EGTA and 10% D_2O to provide a lock signal. The sample temperature was 25 °C, and 5-mm NMR tubes were used. A total of 512 acquisitions were obtained with a spectral width of 4000 Hz, using 16K data points. Broadband proton decoupling was applied during acquisition. The free induction decay was apodized with a single exponential resulting in a line broadening of 1.0 Hz. Chemical shifts were measured relative to external 85% phosphoric acid.

Results

Physical Properties

Ultraviolet Spectral Properties. The UV absorption spectrum and redox properties of the dihydronicotinamide nucleotides are listed in Table I. The λ_{max} of the dihydronicotinamide chromophore is 338 nm in α-araNADH and 346 nm in β-araNADH. As can be seen in Table I, this pattern is opposite to that observed for the ribo coenzymes, where for α-NADH the λ_{max} is 346 nm and for β-NADH it is 338 nm. The extinction coefficients of the dihydronicotinamide moiety are within 3% those of the corresponding ribonucleotides, based on the ratio A_{340}:A_{259}.

Table I: Physical Properties of Ribo and Arabino Coenzymes

	λ_{max} (nm)	redox potential (mV)
β-NADH	338	−320[a]
α-NADH	346	−340[b]
β-araNADH	346	−339
α-araNADH	338	−319

[a] Values from Burton and Wilson (1953) and Rodkey (1955). [b] Values from Kaplan (1960).

Table II: Chemical Shifts[a] and Coupling Constants[b] of Dihydronicotinamide Protons

	N2	N6	N5	$N4_R$	$N4_S$	$\Delta\delta_{R,S}$
β-araNADH	6.824	5.900	4.724	2.843	2.713	0.130
α-araNADH	6.877	5.913	4.801	2.749	2.793	−0.044
	$^3J_{5-6}$	$^3J_{4R-5}$	$^3J_{4S-5}$	$^4J_{4-6}$	$^4J_{6-2}$	$^2J_{4-4}$
β-araNADH	8.2	3.1	3.8	1.4	1.6	−19.2
α-araNADH	7.2	3.6	3.3	2.2	1.6	−18.9

[a] Chemical shifts are reported in parts per million from internal TSP and were obtained at 500 MHz at 20 °C for 1 mM solutions. [b] Coupling constants are in hertz and are reported to the nearest 0.1 Hz.

Redox Potential. The redox potentials for α-araNAD$^+$ and β-araNAD$^+$ have been measured with yeast ADH and the ethanol/acetaldehyde couple according to the procedure of Kaplan et al. (1956). The values of the redox potential obtained under standard conditions are listed in Table I along with the literature values for α-NAD$^+$ (Kaplan, 1960) and β-NAD$^+$ (Burton & Wilson, 1953; Rodkey 1955). The redox potentials for the corresponding α- and β-anomers of the ribo and arabino analogues show an interesting correlation. The value for β-riboNAD$^+$ corresponds to that of α-araNAD$^+$, whereas α-riboNAD$^+$ has the same potential as β-araNAD$^+$.

Anomeric Stability. A unique feature of the dihydronicotinamide ribonucleotides is the configurational instability of the dihydronicotinamide–ribosyl linkage (Woenckhaus & Zumpe, 1965; Oppenheimer et al., 1971). The linkage in NADH anomerizes at measurable rates under physiological conditions (Oppenheimer & Kaplan, 1975), and the reaction can be observed directly by UV spectroscopy (Oppenheimer, 1986a). We find that the dihydronicotinamide arabinonucleotides also anomerize under similar conditions. Both the ribo and arabino coenzymes undergo acid-catalyzed anomerization. For araNADH at 90 °C, a 5:1 ratio of α- to β-anomers, i.e., trans to cis configurations of the base and the 2′-hydroxyl, is observed (see Figure 3). At 22 °C the ratio α:β is 7.5:1, similar to that for NADH (Oppenheimer, 1982). Note that, in contrast to riboNADH, where the β-anomer predominates, the α-anomer is favored for araNADH.

NMR Spectral Parameters

Assignments. The α- and β-anomers of araNAD$^+$ give well-resolved ^1H NMR spectra at 500 MHz as shown in Figure 1. The assignments of the proton resonances have been made by comparison with the resonances of riboNAD$^+$ and riboNADH (Oppenheimer, 1982) and confirmed, where necessary, by spin decoupling experiments. The stereochemical assignments of the N4 protons have been made by enzymatic deuterium labeling. The chemical shifts and coupling constants for the dihydronicotinamide protons are listed in Table II.[3] The values of the chemical shifts and coupling constants have

[3] Complete ^1H NMR spectral data are available in the microfiche addition of the journal (see paragraph at end of paper regarding supplementary material).

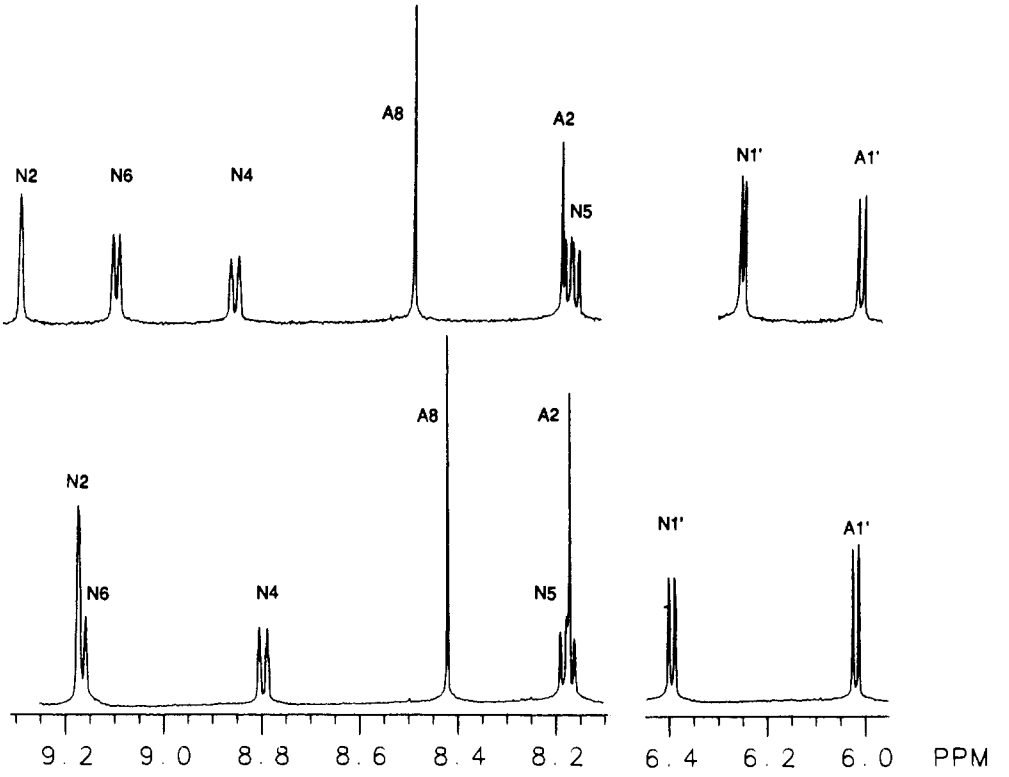


FIGURE 1: Comparison of the downfield region of 500-MHz ^{1}H NMR spectra of 1 mM α- and β-araNAD$^+$ obtained at 20 °C, pD 7.8.

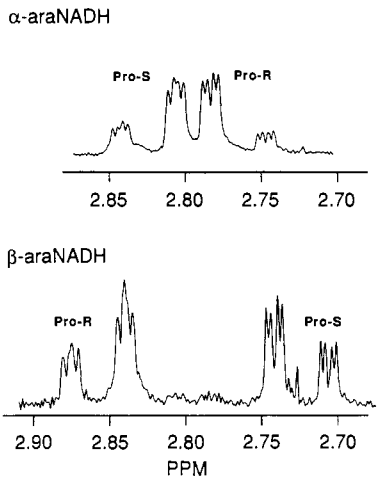


FIGURE 2: Comparison of N4 methylene proton resonances in 500-MHz ^{1}H NMR spectra of α- and β-araNADH obtained at 20 °C. The absolute assignments of the protons are based on enzymatic deuterium labeling experiments.

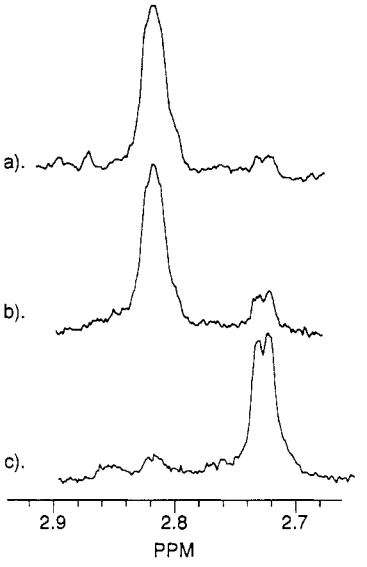


FIGURE 3: Comparison of N4 proton resonances in 360-MHz ^{1}H NMR spectrum of specifically labeled araNADH generated by yeast ADH reduction (a *pro-R*-specific dehydrogenase) with [U-^{2}H]ethanol. Spectrum a is the *pro-S* proton of α-[(*R*)-N4-^{2}H]araNADH, spectrum c is the *pro-S* proton of β-[(*R*)-N4-^{2}H]araNADH, and spectrum b was obtained after the sample of β-[(*R*)-N4-^{2}H]araNADH (shown in spectrum c) was incubated for 5 min at 90 °C. Under these conditions the sample anomerizes to an equilibrium mixture of 5:1 α- to β-anomers (the *S* proton for β-[(*R*)-N4-^{2}H]araNADH can be seen as the upfield doublet). The coincidence of the chemical shifts and coupling constants in spectra b and a confirm that yeast ADH reduces both the α- and β-anomers with identical stereochemistry.

been refined by computer simulation using the program NICSIM.

Chemical Shift Nonequivalence of N4 Methylene Proton Resonances. The nonequivalence of the resonances for the N4 methylene protons is one of the most striking features of ^{1}H NMR spectra of riboNAD(P)H [for a review see Oppenheimer (1982)]. The N4 methylene protons of the α- and β-anomers of araNADH are also nonequivalent as can be seen in the spectra obtained at 20 °C shown in Figure 2. The values of the chemical shifts and the nonequivalence of these protons are listed in Table II. The individual N4 resonances in α- and β-araNADH have been assigned by the following method. A specific deuterium label has been incorporated by using the *pro-R* specific enzyme, yeast ADH, and [U-$^2H_5$]-ethanol in analogy to the procedure outlined by Oppenheimer et al. (1971). A portion of the ^{1}H NMR spectra of the resulting labeled analogues are shown in Figure 3. Note that, for β-araNADH, it is the downfield proton resonance that is eliminated by deuteriation, whereas in the spectrum of labeled α-araNADH the upfield proton resonance is eliminated. Therefore the relative chemical shifts of the N4 protons for α- and β-araNADH are opposite.

The stereochemistry of oxidation of α-NADH by yeast ADH has been shown to be identical with that for β-NADH

Table III: Coupling Constants for the Arabinose Moiety[a]

	1′–2′	2′–3′	3′–4′	4′–5′	4′–5″	5′–5″	5′–P	5″–P
β-araNAD$^+$	6.1	7.8	7.8	2.5	2.5	−11.8	(5.5)	(5.5)
β-araNADH	6.6	7.0	8.1	2.9	2.8	−11.6	3.9	5.8
α-araNAD$^+$	4.2	5.5	5.6	3.1	5.7	−11.8	5.4	5.7
α-araNADH	7.4	8.0	8.5	2.1	5.2	−11.2	4.8	5.2

[a] The coupling constants are in hertz and are reported to the nearest 0.1 Hz. Coupling constants for which only the mean value can be determined because of the fortuitous chemical shift equivalence of the N5′ methylene protons or the pyrophosphate ^{31}P resonances are inclosed in parentheses.

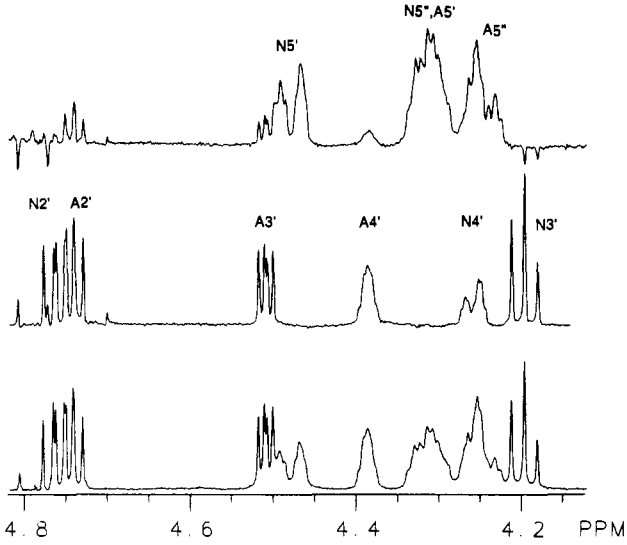


FIGURE 4: Portion of resolution-enhanced 500-MHz ^{1}H NMR spectra of the sugar region of 1 mM β-araNAD$^+$ obtained in D_2O at 20 °C (bottom). The top spectrum is a partially relaxed Fourier transform spectrum in which the methine resonances have been nulled (τ = 1.00 s), and the middle spectrum is a partially relaxed Fourier transform spectrum in which the methylene resonances have been nulled (τ = 0.35 s).

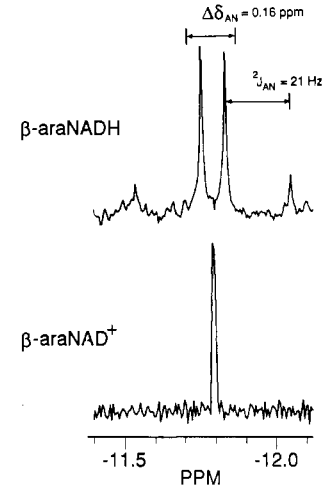


FIGURE 5: 97.3-MHz ^{31}P NMR spectra of 1 mM β-araNAD$^+$ and β-araNADH obtained at 25 °C, pH 7.8. The chemical shifts are referenced to external 85% phosphoric acid.

Table IV: ^{31}P Chemical Shifts of NAD and Related Nucleotides[a]

nucleotide	δ_1	δ_2	$\Delta\delta_{NA}$
β-araNADH	−11.72	−11.88	0.16
β-araNAD$^+$	−11.80	−11.80	<0.004[b]
α-araNADH	−11.62	−11.89	0.27
α-araNAD$^+$	−11.98	−12.14	0.16

[a] Spectra were obtained at 97.3 MHz and are reported in parts per million from external 85% phosphoric acid. Specific assignments of the individual phosphorus resonances have not been made, and $\Delta\delta_{NA}$ is the difference in chemical shift of the two phosphorus resonances. [b] Maximum nonequivalence for the pyrophosphate resonances as calculated from the second-order effects observed on the ^{1}H NMR spectrum at 500 MHz.

through use of the anomerization reaction (Oppenheimer & Kaplan, 1975). The stereochemistry of the yeast ADH catalyzed redox reactions with either the α- or β-anomer of araNAD$^+$ can be established in a similar manner. Starting with the specific label at N4 for β-araNADH, anomerization interconverts the asymmetric center at the 1′-carbon without any alteration in the stereochemistry at the N4 position. As shown in Figure 3, incubation of an anaerobic sample of stereospecifically labeled β-[(*R*)-N4-^{2}H]araNADH at 90 °C for 5 min affords the equilibrium mixture containing 5:1 α- to β-anomers of araNADH. The resulting ^{1}H NMR spectrum of the labeled α-araNADH generated by anomerization of *R*-labeled β-araNADH is identical with the ^{1}H NMR spectrum of the labeled α-araNADH obtained from the direct reduction of α-araNAD$^+$. This result conclusively establishes that the stereospecificity of enzymatic reduction by yeast ADH is identical for both the α- and β-anomers of araNAD$^+$. Therefore we can make the absolute stereochemical assignment of the methylene protons (see Figure 2 and Table II). Note that by establishing the stereochemistry of yeast ADH we can generate specifically labeled arabino coenzymes and thus determine the stereochemistry of araNAD$^+$ in redox reactions catalyzed by other dehydrogenases in analogy to the methodology for riboNAD$^+$ (You et al., 1978).

Analysis of ^{1}H NMR Spectra of Sugar Protons. A portion of the ^{1}H NMR spectrum containing the sugar resonances of β-araNAD$^+$ is shown in Figure 4. The ^{1}H resonances of the arabinose protons for both α- and β-anomers of araNAD$^+$ and araNADH have been assigned by homonuclear spin decoupling experiments and partially relaxed ^{1}H NMR spectra. The coupling constants are listed in Table III. In general the chemical shift properties are similar to the corresponding protons in the ribo coenzymes; e.g., the N1′ proton is shifted 1.387 ppm upfield upon reduction of the β-arabino coenzyme and 1.300 ppm for the β-ribo coenzyme (Oppenheimer, 1982). The spectral properties for the proton resonances of the adenosine moiety show negligible differences from the corresponding resonances in the ribo coenzymes.

^{31}P NMR of the Pyrophosphate Backbone. The ^{31}P NMR spectra of the anomers of araNAD$^+$ and araNADH have been obtained at 97.3 MHz, and the spectra for the β-anomers are shown in Figure 5. Note that the pyrophosphate resonances of β-araNAD$^+$ appear as a singlet. The nonequivalence of the two phosphorus resonances has been calculated to be less than 0.004 ppm, based on computer simulation of the ^{31}P NMR spectrum and the ^{1}H NMR spectrum of the sugar proton resonances. In contrast, the ^{31}P resonances of the pyrophosphate backbone of β-araNADH differ by 0.16 ppm. The ^{31}P resonances in the α-anomers are nonequivalent for both oxidized and reduced forms. The ^{31}P chemical shifts for all four arabino analogues are listed in Table IV.

Enzyme Properties. The α- and β-arabino analogues have been investigated as coenzymes with yeast and horse liver alcohol dehydrogenases and have been found to follow Michaelis–Menten kinetics. The kinetic parameters for araNAD$^+$/NADH with yeast alcohol dehydrogenase are listed

Table V: Kinetic Parameters for Yeast[a] and Horse Liver[b] Alcohol Dehydrogenases

Yeast Alcohol Dehydrogenase			
	V_{max}(rel)	K_M (mM)	V/K (mM^{-1})
β-NAD^+	1.0	0.13	7.7
β-araNAD$^+$	0.03	0.66	0.05
α-araNAD$^+$	7.0×10^{-4}	1.05	6.7×10^{-4}
β-NADH	1.0	0.18	5.5
β-araNADH	0.35	0.50	0.7
α-araNADH	0.001	0.34	2.9×10^{-3}
Horse Liver Alcohol Dehydrogenase			
	V_{max}(rel)	K_M (μM)	V/K (μM^{-1})
β-NADH	1.0	3.0	0.33
β-araNADH	6.1	15.0	0.41
α-araNADH	1.7	59.0	0.03

[a] Assays conducted at 30 °C at pH 8.0 in 50 mM Hepes buffer with saturating ethanol or acetaldehyde (0.1 M). [b] Assays conducted at 30 °C at pH 8.0 in 50 mM Hepes buffer with saturating 1-propanol (0.1 M).

Table VI: Primary Kinetic Isotope Effects for Yeast Alcohol Dehydrogenase[a]

	$^DV_{max}$
β-NAD^+	1.6 ± 0.2
β-araNAD$^+$	5.4 ± 0.7
α-araNAD$^+$	5.3 ± 1.2

[a] Assays conducted at 30 °C at pH 8.0 in 50 mM Hepes buffer with saturating [U-$^2H_5$]ethanol (0.1 M).

in Table V. The relative V_{max} for coenzyme oxidation of β-araNADH is 35% that for β-NADH, whereas the relative V_{max} for reduction of β-araNAD$^+$ is only 3% that for β-NAD^+. As might be expected, the α-anomers are poorer coenzymes. The V_{max} for oxidation of α-araNADH is only 0.1% the rate for β-NADH, and reduction of α-araNAD$^+$ is only 0.07% the rate for β-NAD^+. The results demonstrate that under the assay conditions yeast ADH is better at catalyzing the oxidation of the coenzyme analogues than their reduction. Finally, yeast ADH discriminates between the α- and β-anomers by a factor of 350 for coenzyme oxidation and 43 for coenzyme reduction.

Primary kinetic isotope effects for coenzyme reduction have been measured, and the values are listed in Table VI. Yeast ADH already shows a primary isotope effect of 1.6 for the reduction of β-NAD^+, indicating that hydride transfer is partially rate limiting. Reduction of the α- and β-anomers of araNAD$^+$ shows kinetic isotope effects of 5.3 and 5.4, respectively. Therefore hydride transfer has become fully rate limiting for the analogues.

Enzymatic kinetic parameters have also been measured for horse liver alcohol dehydrogenase, and the values for coenzyme oxidation are listed in Table V. The results are most dramatic. The relative V_{max} for both α- and β-araNADH with horse liver ADH are greater than those for the natural coenzyme β-NADH. The V_{max} for β-araNADH oxidation is 6 times faster than that for β-NADH, and the V_{max} for α-araNADH oxidation is 1.7 times faster. The increase in V_{max} with β-araNADH is accompanied by a 5-fold decrease in the K_m relative to that for β-NADH, giving a V/K value for β-araNADH 20% greater than that for β-NADH. The 20-fold decrease in K_m of α-araNADH still leads to a V/K value that is 10% that for β-NADH.

Discussion

Physical Properties. The differences in the λ_{max} and redox potential listed in Table I for the α- and β-forms of the ribo and arabino coenzymes are most striking. These results establish the sensitivity of the physical–chemical properties of the dihydronicotinamide ring to alterations in the sugar moiety. We see that the redox potential and λ_{max} for the arabino and ribo coenzymes are correlated with the orientation of the 2′-hydroxyl and not with the anomeric configuration of the glycosyl linkage. In dinucleotides where the 2′-hydroxyl is trans to the base (both β-NAD^+ and α-araNAD$^+$), the redox potentials are comparable, −320 and −319 mV, respectively. In contrast, dinucleotides where the 2′-hydroxyl is cis to the base (α-NAD^+ and β-araNAD$^+$) are stronger reductants with redox potentials of −340 and −339 mV, respectively. These effects can be explained by a combination of two factors: (i) destabilization of the electron-rich dihydronicotinamide ring through its juxtaposition with the 2′-hydroxyl and (ii) stabilization of the cationic nicotinamide moiety by the electron-rich 2′-hydroxyl. The results we observe for the ribo- and arabinonucleotide are in general accord with previous studies by Hajdu and Sigman (1977). In their investigations of neighboring group effects on the redox properties of model dihydronicotinamides, they found that the reactivity of the model compounds was best explained by stabilization of the cationic form. For the nicotinamide nucleotides, however, further experiments will be needed in order to determine the individual contributions from these two possibilities.

The K_{eq} for anomerization of dihydronicotinamide nucleotides also shows a correlation with the configuration at C2′. The observed anomeric preferences are consistent with our previous suggestion that steric hindrance and electrostatic interactions between the cis substituents on sugar carbons 1′ and 2′ are the prime determinants of the preferred anomeric configuration (Oppenheimer & Kaplan, 1975). In the cis configuration the bulky, electron-rich dihydronicotinamide ring is juxtaposed with the 2′-hydroxyl. This configuration should be sterically crowded and thus disfavored relative to the less hindered trans orientation. The similarity of the ratio of the respective cis and trans anomers in the ribo and arabino analogues indicates a negligible influence on anomeric equilibria due to stereoelectronic effects between the furanose ring oxygen lone pairs and the C1′ substituents.

Conformation of the Dihydronicotinamide Ring. The ^1H NMR spectra of the N4 methylene protons in riboNAD(P)H have a number of important properties that are consistent with a specific puckering of the dihydronicotinamide ring in the dinucleotide when it is folded against the adenine moiety, including chemical shift nonequivalence of the methylene protons, dependence of the chemical shift nonequivalence on concentration, temperature, and solvent, and differences in the values of the vicinal coupling constants for the N5 and N4 protons (Oppenheimer et al., 1971; 1978; Oppenheimer, 1982, 1986a).

The resonances of the dihydronicotinamide ring in araNADH also show large temperature- and solvent-dependent chemical shifts (data not shown) that are similar to those of riboNADH (Oppenheimer et al., 1978). These results indicate that there are significant contributions from conformations in which the adenine and dihydronicotinamide moieties are based stacked in the arabino analogues. An important and sensitive indicator of the conformation of the dihydronicotinamide ring is the ^1H homonuclear coupling constants (see Table II). The values of the coupling constants of the dihydronicotinamide ring in β-araNADH are similar to those obtained for the β-ribo coenzyme (Oppenheimer et al., 1978). The coupling constant data, combined with the similar order of shielding of the N4 methylene protons of β-NADH and

β-araNADH, argue strongly that both molecules adopt a similar folded form in solution as would be expected from the arguments given by Oppenheimer (1982, 1986a). The results are in accord with a single type of folded conformer being responsible for the preferential distortion of the dihydronicotinamide ring from planarity, and such a form would be in fast exchange with an open, extended form. The similarity of the magnitude of the coupling constants also argues that the degree of distortion is comparable for both the β-ribo and β-arabino coenzymes.

The spectral properties of the dihydronicotinamide resonances for α-araNADH differ fundamentally. The chemical shifts of these resonances clearly indicate that there are major contributions from folded conformations to the solution structure of α-araNADH. On the basis of the coupling constants listed in Table II, however, it is clear that the extent of preferential distortion of the dihydronicotinamide ring is smaller. The near equivalence of the $^3J_{4R-5}$ and $^3J_{4S-5}$ coupling constants indicate that the ring has a more nearly time-averaged planar conformation. These results, combined with the opposite order of the chemical shifts for the N4 protons of α-araNADH (the *pro-S* proton is downfield and the *pro-R* proton is upfield), suggest that there are major contributions from multiple folded forms. Furthermore, these interactions involve both faces of the dihydronicotinamide ring and may involve significantly different relative positioning of the two rings.

Sugar Conformation of Arabino Analogues of Pyridine Nucleotides. The sugar moieties of the α- and β-anomers of the ribonucleotides show distinct redox state dependent conformational changes (Oppenheimer & Kaplan, 1976). In the β-ribonucleotide there is an intramolecular electrostatic attraction between the cationic nicotinamide moiety and the anionic 5′-phosphate. In the α-ribonucleotide the electrostatic interaction is between the base and the *cis*-2′-hydroxyl. Because of the altered stereochemistry of the 2′-hydroxyl, the arabinonucleotides provide an important system to investigate the relative contribution from these interactions in determining sugar conformations.

The value of $J_{3'-4'}$ alone can be used to obtain a qualitative estimate of the population distribution for both ribo- and arabinonucleotides. As discussed by Ekiel et al. (1979) and de Leeuw and Altona (1982), the value of $^3J_{3'-4'}$ should be equally sensitive to the population of sugar conformers for either ribo- or arabinonucleotides. For example, values of $^3J_{3'-4'}$ that are less than 3 Hz indicate a predominance of the an S-type conformer (2′-*endo*) whereas values greater than 7 Hz reflect a predominance of the N-type conformer (3′-*endo*). As shown in Table III, the value of $J_{3'-4'}$ (7.8 Hz) for the β-anomer of araNAD$^+$ undergoes little change upon reduction to β-araNADH where the corresponding value is 8.1 Hz. Larger changes are observed in the values of $J_{3'-4'}$ in α-araNAD$^+$ and α-araNADH, which are 5.6 and 8.5 Hz, respectively. In all cases we see a predominance of the N-type conformers for the nicotinamide arabinonucleotides in contrast to the ribonucleotides, which favor the S-type conformers (Altona & Sundaralingam, 1973).

The favoring of the N-type conformer in the arabinonucleotides is consistent with maintenance of a trans geometry for the 2′ and 3′ diol hydroxyls. Yet unlike the corresponding ribonucleotides there are no simple redox-dependent conformational changes. This result is significant because in the β-arabinonucleotides the base is cis to the 5′-hydroxymethyl group, as found in the β-ribonucleotides, as well as cis to the 2′-hydroxyl, as found in the α-ribonucleotides, i.e., both cases where redox-dependent conformational changes are observed in the ribonucleotides. The reasons for these distinct differences between ribo and arabino nicotinamide nucleotides remain to be investigated.

Conformation of the Pyrophosphate Backbone. The chemical shifts of ^{31}P resonances are especially sensitive to two factors, electrostatic interactions and the conformation of vicinal substituents. The ^{31}P NMR spectra of the ribo coenzymes have been reported (Blumenstein & Raftery, 1972; Sarma & Mynott, 1972), and their results demonstrate that the two ^{31}P resonances of the pyrophosphate of NAD$^+$ are well resolved at 87 MHz. The spectrum of β-NAD$^+$ appears as an A–B pattern with a $\Delta\delta_{NA}$ of 0.29 ppm and a geminal coupling constant of −20 Hz. In contrast, the ^{31}P NMR spectrum of β-NADH appears as a single peak in which the individual resonances cannot be resolved. The nonequivalence of the ^{31}P resonances in β-NAD$^+$ but not β-NADH has led to the proposal that the difference in chemical shift of the pyrophosphate resonances is due to an electrostatic interaction between the pyridinium cation and the anionic pyrophosphate backbone (Blumenstein & Raftery, 1972). By this reasoning, in the reduced form the absence of the charged pyridinium would lead to similar chemical environments for the two ^{31}P resonances. That is, the adenine and dihydronicotinamide rings are considered to have equivalent electrostatic properties; thus they should impart similar chemical shift environments on the ^{31}P resonances.

The electrostatic model, however, is not consistent with the results for the arabino analogs of NAD. As can be seen in Figure 5, the pyrophosphate resonances of β-araNAD$^+$ are less than 0.004 ppm nonequivalent whereas in β-araNADH they differ by 0.15 ppm. Clearly then, the arabinonucleotides, unlike the ribonucleotides, show an opposite correlation between the charge of the heterocyclic ring and the chemical shift nonequivalence of the two phosphorus resonances. Therefore, electrostatic interactions are not the sole factor defining the magnetic environment of the pyrophosphate backbone.

Model studies by Gorenstein (1984) have shown that the chemical shifts of ^{31}P nuclei are as sensitive to the conformation of substituents as they are to electrostatic effects such as protonation of the phosphate. Comparison of the values of $J_{4'-5'R}$ and $J_{4'-5'S}$ listed in Table II to the previously published data for the ribo coenzymes (Oppenheimer, 1982) indicate that the rotamer populations around the 4′–5′ and 5′–O bonds are similar for both the ribo and arabino coenzymes. Therefore, significant differences in rotamer populations alone can not account for the contrasting behavior of the ^{31}P resonances of the pyrophosphate backbone for the two sets of compounds.

The sensitivity of the ^{31}P chemical shift to conformational changes may mean that the observed patterns of chemical shifts are in response to subtle differences in the rotamer populations. Note that the values of $\Delta\delta_{NA}$ are less than 0.2 ppm whereas the range of conformational-dependent shifts can be as large as 3 ppm, suggesting that differences of less than 10% in the rotamer populations could account for the effects. Alternatively there may be differences in the correlation of the rotamers around the 4′–5′ and 5′–O bonds. As discussed by Oppenheimer and Kaplan (1976), the populations of the overall orientations can change in response to alterations of the total interaction potential even in the absence of any significant changes in the values of the individual rotamer populations. Such changes in correlation could easily account for the attendant changes in the chemical shifts of the ^{31}P resonances without any changes in the values of the observed vicinal coupling constants.

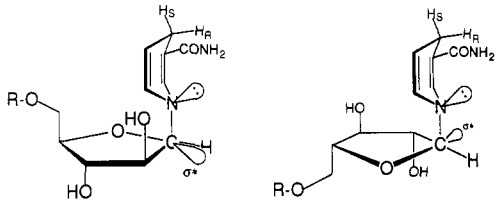


FIGURE 6: Schematic representation showing orientations of the lone pair of electrons on the ring nitrogen relative to the C1′–O antibonding orbital, σ^*, for α- and β-configurations of reduced pyridine coenzymes.

Enzyme Activity. The ability of both β-araNAD$^+$ and α-araNAD$^+$ to serve as coenzymes for yeast and horse liver ADH demonstrates conclusively that specific interactions between the enzyme and the 2′-OH, or for that matter the entire sugar moiety, are not *essential* in an absolute sense for their ability to function as coenzymes. The activity of the α-anomer is all the more remarkable if one considers the large differences in the spatial arrangement of the substituents around the anomeric carbon and the generally acknowledged high degree of stereoselectivity shown in most enzyme–substrate interactions and their resulting chemical reactions. The magnitude of the differences between α- and β-anomers is shown in Structure I. Here we see explicitly the dissimilar disposition of substituents for the α-anomer of araNAD$^+$ relative to either β-araNAD$^+$ or NAD$^+$. Note two important distinctions between the binding of α-anomers and that of β-anomers. First, in the β-anomers the sugar hydroxyls are directed toward the surface of the protein and the less crowded furanose ring oxygen is seated toward the protein. Second, the 5′-hydroxymethyl group and its attached pyrophosphate linkage is cis to the base. In the α-anomers these arrangements have to be altered drastically in order for the nicotinamide ring to be seated for proper stereochemical function. The hydroxyls now must be directed toward the protein, and the 5′-phosphate is trans to the nicotinamide ring. Therefore our results suggest that the active site is designed to *accommodate* the sugar moiety sterically rather than to *bind* it via specific interactions.

The observed activity of both the α- and β-anomers of the ribo and arabino pyridine coenzymes also has a direct bearing on recent proposals regarding the mechanism of dehydrogenases. Benner (1982) and co-workers (Nambiar et al., 1983) have proposed a correlation between the stereochemistry of enzyme-catalyzed hydride transfer and the external redox potential of the substrates for a group of alcohol dehydrogenases meeting certain stringent criteria. The theoretical rationale for their correlation is a proposed stereoelectronic effect involving a specific interaction of the lone pair of the N1 nitrogen of the dihydronicotinamide ring and the antibonding orbital σ^* of the C1′–O bond. Since both α-riboNADH (Oppenheimer & Kaplan, 1975; Oppenheimer, 1986b) and α-araNADH transfer hydride with the stereochemistry identical with that found from β-riboNADH, then the seating of the dihydronicotinamide ring with respect to the substrate in the active site must be the same in all cases. Given the importance of the adenosine moiety for coenzyme binding, it is also reasonable that the ADP group of these analogues binds in its usual site. An antiperiplanar orientation between the O′–C1′ bond and the lone pair on the ring nitrogen is readily achieved in the β-anomer as shown in Figure 6. However, an α-anomer can not achieve an antiperiplanar geometry without a 120° rotation around the N–C1′ glycosyl bond. Such a rotation no longer permits the alignment of the dihydronicotinamide ring in the actve site that is necessary to satisfy the observed stereospecificity of enzyme-catalyzed hydride transfer. As a consequence, any specific orbital interactions that occur for the β-anomers, even if not explicitly directed by the enzyme, must be either nearly eliminated or even reversed when the enzyme binds and utilizes an α-anomer. Since yeast ADH is an enzyme that fully meets the criteria as set forth in their paper (Nambiar et al., 1983), it is then apparent that a specific alignment of the designated electron orbitals is not *essential* for catalysis. These results raise additional questions regarding the general validity of their proposal (Oppenheimer, 1984).

Although the studies of the ability of the arabino analogues of NAD$^+$ to function as a coenzyme analogue have focused on yeast and horse liver ADH preliminary results indicate that these properties are shared by a number of other common dehydrogenases. The ability of dehydrogenases to utilize coenzyme analogues with significant differences in substituents on the sugar moiety will have important consequences. This property should allow the design of other new analogues to investigate steric requirements for binding, specific mechanistic questions, and substrate–coenzyme interactions for a wide range of dehydrogenases and other pyridine coenzyme dependent enzymes.

Supplementary Material Available

Three tables showing complete ^{1}H NMR data for α- and β-araNAD$^+$ and α- and β-araNADH (1 page). Ordering information is given on any current masthead page.

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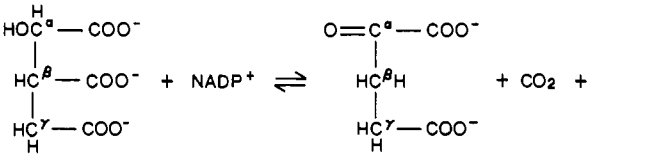
Ionization of Isocitrate Bound to Pig Heart $NADP^+$-Dependent Isocitrate Dehydrogenase: ^{13}C NMR Study of Substrate Binding†

Robert S. Ehrlich and Roberta F. Colman*
Department of Chemistry, University of Delaware, Newark, Delaware 19716
Received November 6, 1986; Revised Manuscript Received January 20, 1987

ABSTRACT: Isocitrate and α-ketoglutarate have been synthesized with carbon-13 enrichment at specific positions. The ^{13}C NMR spectra of these derivatives were measured as a function of pH. The magnitudes of the changes in chemical shifts with pH for free isocitrate and the magnesium–isocitrate complex suggest that the primary site of ionization is at the β-carboxyl. In the presence of the enzyme $NADP^+$-dependent isocitrate dehydrogenase and the activating metal magnesium, the carbon-13 resonances of all three carboxyls remain constant from pH 5.5 to pH 7.5. Thus, the carboxyls remain in the ionized form in the enzyme–isocitrate complex. The α-hydroxyl carbon resonance could not be located in the enzyme–isocitrate complex, suggesting immobilization of this group. Magnesium produces a 2 ppm downfield shift of the β-carboxyl but does not change the resonances of the α- and γ-carboxyls. This result is consistent with metal activation of both the dehydrogenation and decarboxylation reactions. The ^{13}C NMR spectrum of α-ketoglutarate remains unchanged in the presence of isocitrate dehydrogenase, implying the absence of alterations in geometry in the enzyme-bound form. Formation of the quaternary complex with Mg^{2+} and NADPH leads to loss of the α-ketoglutarate resonances and the appearance of new resonances characteristic of α-hydroxyglutarate. In addition, a broad peak ascribed to the enol form of α-ketoglutarate is observed. The substantial change in the shift of the β-carboxyl of isocitrate and the lack of significant shifts in the other carboxyls of isocitrate or α-ketoglutarate suggest that interaction of the β-carboxyl with the enzyme contributes to the tighter binding of isocitrate and may be significant for the oxidative decarboxylation function of isocitrate dehydrogenase.

The oxidative decarboxylation of isocitrate to form α-ketoglutarate by $NADP^+$-dependent isocitrate dehydrogenase from pig heart [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] requires a divalent metal (Villafranca & Colman, 1972; Colman, 1983):



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