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Determination of the Absolute Stereospecificity of the Enzymatic Reduction of NAD+ Analogs by Means of the Primary Acid Cyclization Reaction

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NADH, (3-acetylpyridine)ADH, (3-thionicotinamide)ADH and (pyridine-3-aldehyde)ADH were labeled with deuterium by the B specific enzyme lipoyl dehydrogenase. The primary acid rearrangement products, α-$O^{2'}$-6B cyclotetrahydropyridine adenine dinucleotides, of the respective reduced coenzyme analogs were synthesized by incubation at pH 2–2.5. Analysis of the spin–spin coupling constants of the 220 MHz proton magnetic resonance spectra establishes that the structure of the ribose-tetrahydropyridine linkage of these analogs is the same as that of the primary acid product of NADH. All four specifically labeled dinucleotides possess the identical absolute configuration at the C4 position with respect to the deuterium label, establishing that the three coenzyme analogs are reduced by lipoyl dehydrogenase with the same absolute stereospecificity as NAD^+. The primary acid reaction is described as a general method for the determination of the absolute configuration at the C4 position of specifically labeled β-1,3-substituted, 1,4 dihydropyridine nucleotides.

INTRODUCTION

The dehydrogenases are an ubiquitous class of enzymes which catalyze the reversible transfer of hydride from a substrate to the C4 position of the nicotinamide ring of NAD^+.[1] These enzymes can differentiate between the diastereotropically paired hydrogen atoms of the C4 methylene group of NADH and can transfer the hydride to the substrate stereospecifically (*4*). The dehydrogenases have been classified into two distinct groups on the basis of the specificity of the hydride transfer to the C4 position: the A-specific enzymes which transfer hydride to the A side of the nicotinamide ring, and the B-specific enzymes which transfer hydride to the B side (*5*). This classification scheme gave only the relative stereospecificities of the reactions until the absolute

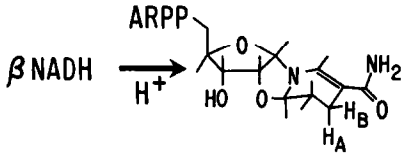
[1] *Abbreviations.* In the present paper we have used the NAD system of nomenclature in order to abbreviate more clearly the various pyridine analogs and modification products. (3-acetylpyridine) AD^+, (AcPy)AD^+; (3-thionicotinamide)AD^+, (TN)AD^+; (Pyridine-3-aldehyde)AD^+, (PA)AD^+. $NADD_B$, (AcPy)ADD_B, (TN)ADD_B and (PA)ADD_B; reduced coenzyme and analogs specifically labelled with deuterium in the pro-S (B) position. $NADD_A$, reduced coenzyme specifically labelled with deuterium in the pro-R (A) position. The pro-R and pro-S nomenclature (1–3) will be used as well as the traditional A and B designation for the faces of the dihydropyridine ring. (cTHN)AD, (cTHAcPy)AD, (cTHTN)AD and (cTHPA)AD are the α-$O^{2'}$-6B-cyclotetrahydropyridine adenine dinucleotides formed by the primary acid rearrangement of NADH, (AcPy)ADH, (TN)ADH, and (PA)ADH, respectively.

stereospecificity of the oxidation/reduction of the pyridine ring was determined chemically by Cornforth *et al.* (*6*, *7*). They established that the A proton of the dihydronicotinamide ring had a pro-R configuration and the B proton a pro-S configuration.

Investigations into the interaction of the coenzyme with dehydrogenases: the binding, chemical mechanism of reduction, inhibition, etc. (*8–10*), as well as studies of the conformation of the coenzyme in solution (*11*) have been greatly aided by the availability of analogs of NAD^+ containing various chemical modifications at the C3 position of the pyridine ring (*12*, *13*). While the analogs are a powerful tool in the study of the dehydrogenases, the possibility of differences in the stereospecificity of the enzymatic reduction of the analogs has not been seriously considered. It has been assumed a priori that the reduction of the analogs would have the identical stereochemistry as NAD^+. Implicit in this assumption is the belief that the enzyme would bind the modified pyridine ring with the same orientation as the nicotinamide ring of NAD^+.

Sarma and Kaplan (*14*) questioned whether all β-1,3-substituted pyridine coenzymes would be reduced with the identical stereospecificity since preliminary proton magnetic resonance (PMR) studies suggested apparent distinct differences in the syn vs anti tortional isomers around the ribosidic bond of certain pyridine analog mononucleotides. As an alternative they proposed that the conformation of the dinucleotide might, in fact, be able to determine the absolute configuration of the deuterium labeling of the pyridine ring in an anzymatic reduction. That is, the stereospecificity of the reduction would depend on whether the enzyme were A or B specific as well as on the analog that was being reduced. This possibility has been raised for a new analog. Biellmann and Jung (see note added in proof) (*15*) have reported that a discrepancy may exist between the reduction of (TN)AD^+ and a new analog (3-cyanopyridine)AD^+ by yeast and horse liver alcohol dehydrogenase. The possibility of altered enzymatic specificity can only be tested by the chemical determination of the absolute configuration of the enzymatically prepared, specific deuterium labeled coenzyme analogs. We propose to determine the absolute stereospecificity of the enzymatic reduction of the pyridine ring of three commonly used NAD^+ analogs: (AcPy)AD^+, (TN)AD^+, and (PA)AD^+ by using a new method, the primary acid rearrangement reaction.

The reaction of the dihydropyridineribose moiety in dilute acid provides a chemical synthesis of an asymmetric center of known absolute configuration at the C6 position (*16*). The series of acid-catalyzed reactions involve, not necessarily in this order, protonation at the C5 position, epimerization to the α anomer, and an attack of the ribose 2′ hydroxyl on the C6B position. These results are summarized in the following equation:



The absolute configuration of a specific deuterium label at the C4 position can be determined from the PMR spectrum of the acid product by a detailed analysis of the spin–spin coupling constants of the $O^{2'}$-6B cyclotetrahydropyridine proton

absorptions. Thus, the stereospecificity of the enzymatic reduction can be easily established and any differences recognized.

METHODS

NAD^+ was obtained from P-L Biochemicals and used without further purification. The analogs $(AcPy)AD^+$, $(TN)AD^+$, and $(PA)AD^+$ were prepared by Mr. F. Stolzenbach and Mrs. C. L. Lee in this laboratory by the brain DPNase exchange method (*17*). Coenzymes $NADD_B$, $(AcPy)ADD_B$, $(TN)ADD_B$, and $(PA)ADD_B$ specifically labeled with deuterium on the B-side were prepared by pig heart lipoyl dehydrogenase (EC 1.6.4.3, Boehringer–Mannheim) reduction in D_2O (*11*).

The primary acid products were prepared by adjusting the pH of an unbuffered, 10 m*M* solution of the reduced coenzyme with 2 *N* HCl to pH 2–2.5 and then incubating the solution until the uv absorption of the dihydropyridine ring decreased to less than 5% of the original value. The acid reaction was monitored at the following wavelengths: 340 nm for $NADD_B$, 358 nm for $(PA)ADD_B$, 363 nm for $(AcPy)ADD_B$, and 395 nm for $(TN)ADD_B$ (*18*). The reaction was quenched by neutralizing with 2 *M* ammonium bicarbonate; the solution was then placed on a DEAE-11 cellulose–bicarbonate column and eluted with a 5 m*M*–500 m*M* ammonium bicarbonate linear gradient. Yields of the primary acid product were better than 90% based on the initial reduced dinucleotide. Thus 10 mg of labeled analog were sufficient to determine the stereospecificity of the enzymatic reduction.

Extinction coefficients were calculated from the spectrum of the primary acid product formed by incubating a known concentration of reduced coenzyme at low pH in a cuvette until no further increase in the tetrahydropyridine absorption was observed. The uv spectra were recorded on a Cary 14 or a Perkin–Elmer Colemann 124 double-beam scanning spectrophotometer.

PMR spectra were obtained on a Varian Associates field sweep HR-220 proton magnetic resonance spectrometer. Signal-to-noise ratio was enhanced with a Nicolet 1074 computer. The samples were twice lyophilized from 99.8% D_2O and run at a concentration of 50 m*M* in 100% D_2O, pD 8.5, and 22°C (the ambient temperature of the probe). Sample volume was 0.2 ml and Teflon vortex plugs (Wilmad) were used. The pD was measured with a Radiometer 25 pH meter using a combination electrode. The standard electrode correction factor was used, pD = meter reading + 0.4 (19). An internal standard, 3 m*M* TSP, trimethylsilyl sodium propionate (tetradeutero), was used and 1 m*M* EDTA was routinely added to suppress any line broadening caused by possible paramagnetic impurities.

RESULTS

The time course of the changes in the uv absorption spectra of the dihydropyridine ring of NADH and the three reduced analogs incubated at low pH is shown in Fig. 1–4. The frequency and extinction coefficients of the absorption maxima of the primary acid

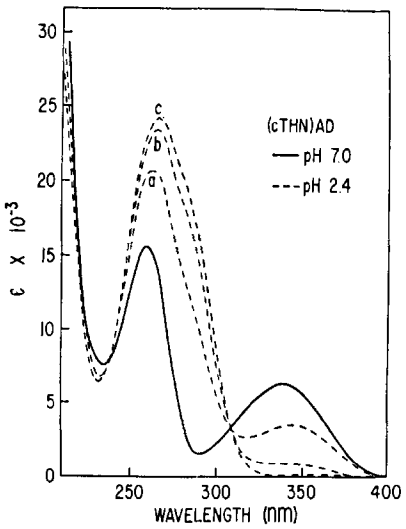


FIG. 1. UV absorption spectra of the reaction NADH to cTHNAD, scanning rate 60 nm/min, time given is for the start of the scan at 400 nm (——) NADH at pH 7. (a) (----) NADH at pH 2.4, 0:20 min. (b) 3:45 min. (c) 10:00 min. No further change observed.

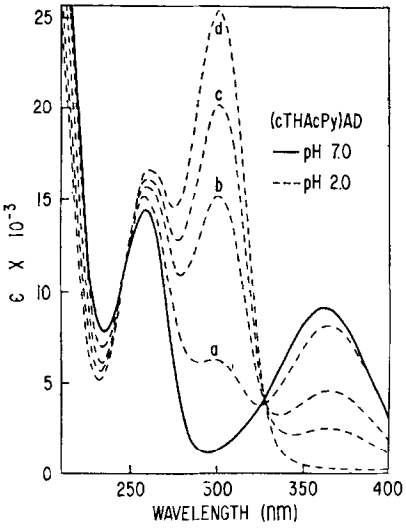


FIG. 2. UV absorption spectra of the reaction (AcPy)ADH to (cTHAcPy)AD, scanning rate 60 nm/min, time given is for the scan at 400 nm (——) (AcPy)ADH at pH 7. (a) (----) (AcPy)ADH at pH 2.0, 0:20 min. (b) 4:30 min. (c) 9:00 min. (d) 21:00 min. No further changes observed.

products are listed in Table 1. The acid product of (TN)ADH, (cTHTN)AD, shown in Fig. 3, has two absorptions, band I at 346 nm and band II at ~285 nm. Analogous absorptions occur in the uv spectrum of (TN)ADH where the main absorption of the dihydrothionicotinamide ring occurs at 395 nm and a shoulder is observed at 298 nm (*18*).

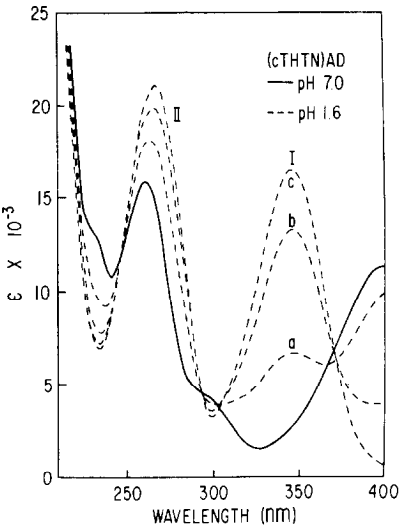


FIG. 3. UV absorption spectra of the reaction (TN)ADH to (cTHTN)AD, scanning rate 60 nm/min, time given is for the start of the scan at 400 nm (——) (TN)ADH at pH 7. (a) (----) (TN)ADH at pH 1.6, 0:20 min. (b) 4:00 min. (c) 16:00 min. No further change observed.

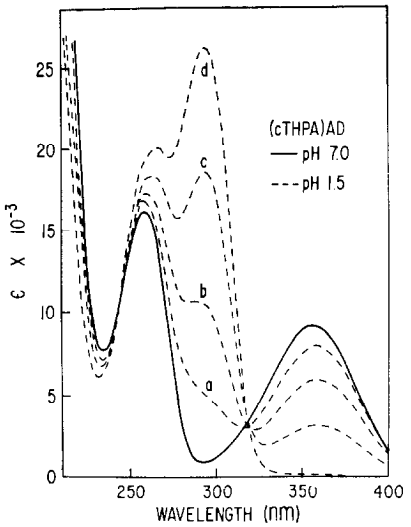


FIG. 4. UV absorption spectra of the reaction (PA)ADH to (cTHPA)AD, scanning rate 60 nm/min, time given is for the start of the scan at 400 nm (——) (PA)ADH at pH 7. (a) (----) (PA)ADH at pH 1.5, 4:00 min. (b) 11:00 min. (c) 24:00 min. (d) 75:00 min. No further change observed.

The dihydropyridine rings of the various analogs have different stabilities with respect to acid; (PA)ADH is the most resistant to the acid reaction, while NADH is the most liable. The same relative order of stability for these four dihydropyridine ribose moieties has been reported for the reactions catalyzed by ionorganic phosphate at pH 6.6 (*20*). As can be seen in Fig. 1–4 the reaction has distinct isobestic points

TABLE 1

EXTINCTION COEFFICIENTS AND ABSORPTION MAXIMA FOR THE ACID PRODUCTS OF THE REDUCED PYRIDINE NUCLEOTIDE ANALOGS

	λ_{max} in nm	$\varepsilon \times 10^{-3}$
(cTHN)AD	282	22.2 (at 266 nm)[a]
(cTHAcPy)AD	303	25.4 (at 303 nm)
(cTHTN)AD	345 I	16.4 (at 345 nm)
	285 II	21.0 (at 267 nm)[a]
(cTHPA)AD	294	25.8 (at 294 nm)

[a] ε for the observed absorption maxima of the combined adenine–cyclotetrahydropyridine chromophores of the acid product.

indicating that there is no significant concentration of intermediates having substantially different absorption characteristics.

PMR studies. The chemical shifts for the proton absorptions of the C4 methylene group of the dihydropyridine ring of NADH and the reduced analogs are given in Table 2. Reduction of the oxidized analogs with [U-^{2}H] ethanol (C_2D_5OH) and the A-specific enzyme yeast alcohol dehydrogenase in all cases eliminates the downfield

TABLE 2

CHEMICAL SHIFT OF THE PC_4 PROTONS[a]

	PC_4H_A	PC_4H_B	$\Delta\sigma^b$
NADH	602.6	580.2	22.4
(AcPy)ADH	561.2	551.4	9.8
(TN)ADH	620.2	598.1	22.1
(PA)ADH	582.7	572.2	10.5

[a] Hz from TSP at 220 MHz, 22°C, ±0.2 Hz.

[b] Difference in chemical shift between the A and B protons.

proton absorption of the C4 methylene group of the dihydropyridine ring (*11*). Reduction in D_2O with the B-specific enzyme lipoyl dehydrogenase eliminates the upfield proton absorption of the C4 methylene group (*11*). In the case of NADH where the absolute configuration has been independently established (*6*) these results lead to the direct assignment of the C4 methylene protons; the downfield absorption is the pro-R or A proton, and the upfield absorption is the pro-S or B proton (*11*). Even though this same pattern of relative chemical shifts is observed for the C4 methylene protons of the other three analogs, it is not sufficient evidence by itself to establish their absolute

configuration. This is because the chemical shift non-equivalence is generated by a time-averaged fast intramolecular association between the dihydropyridine ring and the adenine ring in the dinucleotide (*11*, *21*, *22*). Thus, the non-equivalence is a function not only of the configuration of the C4 position, but of the overall conformation of the dinucleotide as well.

The chemical shifts for the PMR absorptions of the tetrahydropyridine ring of the primary acid product of $NADD_B$, (cTHN)AD($4D_B$), and the specifically labeled analogs are given in Table 3. The proton assignments are based on chemical shift and

TABLE 3

THE CHEMICAL SHIFTS OF THE CYCLOTETRAHYDROPYRIDINE RING[a]

	PC_2H	PC_6H	PC_5He	PC_5Ha	PC_4H_A
(cTHN(AD)$4D_B$)	1592	1098	485	278	450
(cTHAPy)AD($4D_B$)	1683.5	1099.5	490.5	254	396
(cTHTN)AD($4D_B$)	1747	1102.5	497.5	282	455
(cTHPA)AD($4D_B$)	1615.5	1113	500	259	410.5

[a] Chemical shifts in Hz from TSP at 220 MHz, 22°C, ±0.5 Hz.

coupling constants analogous to those found with (cTHN)AD (*16*) and were confirmed by homonuclear spin decoupling experiments. The relative chemical shifts for corresponding protons are maintained. As is the case for the acid product of NADH, only one of the two possible diastereomers expected from the generation of an asymmetric center at the C6 position is detected. In contrast the related compound β-6 hydroxytetrahydronicotinamide adenine dinucleotide, NADHX, is a mixture of the two possible diasteromers (*23*).

The coupling constants of the cyclotetrahydropyridine protons for the acid product of the specifically labeled reduced coenzyme are given in Table 4. The values for the coupling constants of the primary acid products of the analogs show only very minor

TABLE 4

COUPLING CONSTANTS
αO²′-6B CYCLOTETRAHYDROPYRIDINE RING PROTONS[a]

	$J_{6\text{-}5a}$	$J_{6\text{-}5e}$	$J_{5a\text{-}4A}$	$J_{5a\text{-}5e}$	$J_{5e\text{-}4A}$	$J_{4A\text{-}2}$
(cTHN)AD($4D_B$)	9.5	4.2	13.0	−11.7	5.4	1.8
(cTHAcPy)AD($4D_B$)	9.6	4.1	13.5	−12.5	3.4	1.3
(cTHTN)AD($4D_B$)	9.7	4.2	13.0	−12.2	4.7	1.5
(cTHPA)AD($4D_B$)	9.8	4.3	13.5	−12.0	5.2	1.5

[a] The values are accurate to within 0.2 Hz.

differences from the values for (cTHN)AD(4D_B). More importantly, the coupling pattern of the C5ax protons shown in Fig. 5 is identical for all four primary acid products. Likewise, the chemical shifts and coupling constants of the ribose protons for these

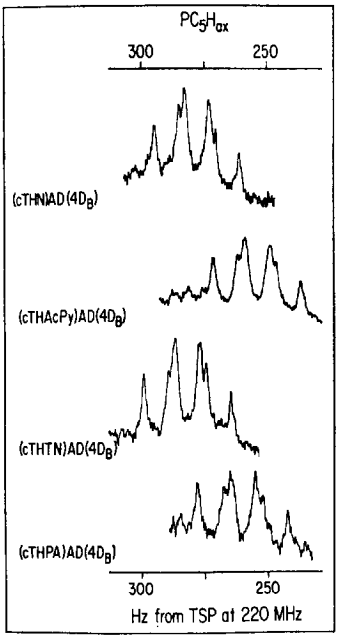


FIG. 5. Portion of the PMR spectra showing the PC_{5ax} protons of the acid products of the specifically labeled $NADD_B$ and analogs.

primary acid products listed in Table 5 demonstrate a close correspondence to the values for the ribose region of (cTHN)AD(4D_B). This similarity in J values is significant because the coupling constants for vicinal protons are a function of the dihedral angle between the carbon-proton bonds, the Karplus relationship (*24*). Thus, the values of the

TABLE 5

COUPLING CONSTANTS AND CHEMICAL SHIFTS FOR THE α-RIBOSE PROTONS

	Coupling constants[a]			Chemical shift[b]		
	$J_{1'-2'}$	$J_{2'-3'}$	$J_{3'-4'}$	$PC_1'H$	$PC_2'H$	$PC_3'H$
(cTHN)AD(4D_B)	4.5	5.4	8.8	1218	1011	891
(cTHAcPy)AD(4D_B)	4.5	5.5	8.5	1240	1029.5	908.5
(cTHTN)AD(4D_B)	4.5	5.6	8.8	1245.5	1025	901
(cTHPA)AD(4D_B)	4.5	5.6	8.6	1246	1038	903

[a] The values are accurate to within 0.2 Hz.

[b] Chemical shifts in Hz from TSP at 220 MHz, 22°C, ±0.5 Hz.

coupling constants are related to the orientation of protons on adjacent carbons and are, therefore, sensitive to any differences which may exist in the conformation of related compounds. The data listed in Tables 4 and 5 are evidence that the primary acid products of all the analogs have an identical geometry, illustrated in Fig. 6. The results for the natural coenzyme and the analogs can be summarized as follows: (1) there are only minor differences between the coupling constants for the corresponding protons of the cyclotetrahydropyridine ring of the analogs; (2) there are no differences, within experimental error, between the coupling constants of the ribose protons; and (3) only one unique set of coupling constants corresponding to the stereospecific formation of a single diastereomer is observed.

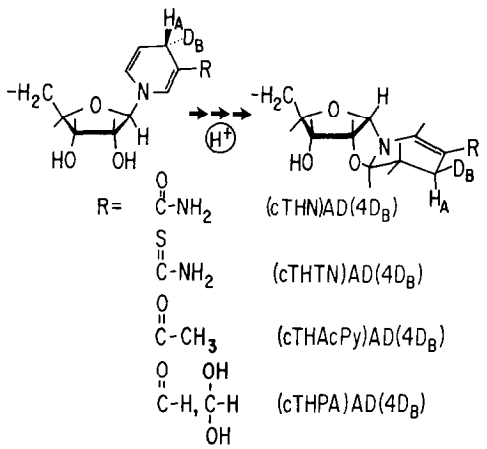


FIG. 6. Structure of the primary acid products.

DISCUSSION

In their PMR studies at 220 MHz Sarma and Kaplan (*14*) observed differences in the effect that the titration of the 5′ phosphate group had upon the protons of the pyridine ring of nicotinamide mononucleotide, NMN^+, and the protons of the (3-acetylpyridine)-mononucleotide, $(AcPy)MN^+$. The C2 proton of NMN^+ shifts 27.5 Hz while the C6 proton shifts only 10.6 Hz upon titration of the 5′ phosphate. For $(AcPy)MN^+$ the C2 proton shifts −2.0 Hz and the C6 proton shifts 37.0 Hz. The predominance of the effect of titration of the 5′ phosphate on the C2 proton of MNN^+ was interpreted as a close juxtaposition of the C2 proton to the 5′ phosphate, hence that the 3-amido group of the nicotinamide ring was oriented syn with respect to the ribose moiety.[2] The preferential shift of the C6 proton in $(AcPy)MN^+$ was interpreted to indicate that the 3-acetyl group was oriented anti. Since it was also postulated that rotation around the ribosidic bond was restricted (*14*), it then seemed possible that this difference in syn or anti conformation of the mononucleotides could occur in their respective dinucleotides as well, both in solution and on the enzyme surface. If this were the case, Sarma and

[2] The results for NMN^+ have recently been interpreted by Sarma and Mynott (*25*) in terms of a two-state syn/anti model.

Kaplan speculated that the 3-acetyl pyridine analog might be labeled "backwards"; i.e., an A-specific dehydrogenase, which would normally incorporate deuterium into the C4 pro-R position of the dihydronicotinamide ring, might label the dihydroacetylpyridine ring in the C4 pro-S position instead, as is shown in Fig. 7.

The determination of the absolute stereospecificity of the reduction of the NAD^+ analogs by only enzymatic means is impossible when questions are raised about the assumption of the similarity of the binding conformations of the analogs to that of NAD^+. Thus, if some A-specific dehydrogenase were to label the NAD^+ analogs

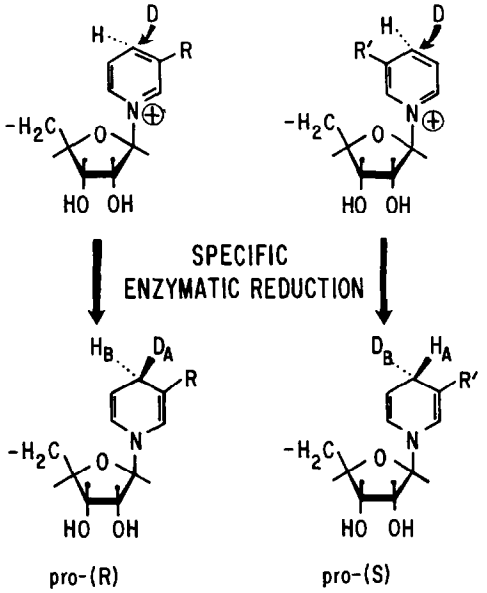


FIG. 7. Mechanism for the possible influence of the NAD^+ analogs conformation on the stereospecificity of the reduction of the pyridine ring by the same enzyme. This figure is not intended to imply a preference for the tortional conformation around the ribosidic bond of NAD^+ in the active site. However, results on the X-ray structure of lactate dehydrogenase (*28*) indicate that the nicotinamide ring is oriented anti in the active site.

"normally" while other A-specific dehydrogenases were to label them "backwards." the situation would be obviously chaotic. The problem could be even subtler if one further assumed that (AcPy)AD^+ were to be labeled "backwards" with respect to NAD^+ in all cases, then even though a self-consistent set of stereospecificity relationships could be established it would only be the relative stereospecificity which would have been determined just as was the situation with NADH (*26*) prior to the work of Cornforth (*6*). Only the direct determination of the absolute configuration at the C4 position can establish whether the enzymatic reduction of the analogs occurs with the same absolute stereospecificity as does the reduction of NAD^+. If such backward labeling were to be demonstrated for (AcPy)AD^+ or any other analog, then this result would have profound consequences regarding the nature of the coenzyme–enzyme

binding interaction and would indicate the overriding importance of the C3 substitution on the conformation of the coenzyme in the active site.

The primary acid reaction of the dihydropyridine-ribose moeity forms a new asymmetric center of known absolute configuration and enables the determination of the absolute configuration of the asymmetric center at the C4 position by analysis of the spin-spin coupling constants of the protons of the cyclotetrahydropyridine ring. (*16*). The ribose 2′ hydroxy adds to the 6B equatorial position hence the C6 proton is axial and on the A side of the cyclotetrahydropyridine ring. The 9.5 Hz value of J_{6-5ax} corresponds to a *trans* conformation, hence the C5ax proton is on the B-side. Using the coupling constant between the 5ax proton and the C4 proton of the specifically labeled primary acid products, it is a straightforward matter to determine the absolute configuration at the C4 position. A value of the J_{5ax-4} coupling constant of 10–13 Hz would indicate that the C4 proton is trans, hence with a pro-R(A) configuration. A value of J_{5ax-4} of 2–6 Hz would indicate a pro-S (B) configuration.

The observation of a large 13–13.5 Hz value for J_{5ax-4} in the primary acid products of the specifically labeled analogs unambiguously assigns the C4 proton to the A-side (pro-R) configuration with the deuterium label having a B-side (pro-S) configuration. Thus, (AcPy)ADD_B, (TN)ADD_B and (PA)ADD_B have the same absolute stereoconfiguration at the C4 position as $NADD_B$. The various C3 substituents on the pyridine ring have no effect on the stereospecificity of lipoyl dehydrogenase reduction of these three coenzyme analogs. The stereospecificity of any other enzymatic reduction of these analogs can be determined relative to lipoyl dehydrogenase by the more standard methods (*5*). Furthermore, the absolute assignment of the C4 methylene proton absorptions of the dihydropyridine ring also enables the stereospecificity of enzymatic reductions to be determined directly from the PMR spectrum of the specifically labeled reduced analogs as well. Thus (AcPy)AD^+ (*11*) and (TN)AD^+ have been found to be reduced with [U-^{2}H] ethanol by yeast alcohol dehydrogenase, an A-specific enzyme, with the identical specificity as that for NAD^+.

The primary acid rearrangement provides a means to determine the configuration at the C4 position. This method is not limited solely to the use of a 220 MHz PMR spectrometer. Lower frequency, 100-MHz, 90-MHz, or even 60-MHz spectrometers can be utilized either with computer enhancement capabilities (computer summing of spectra or Fourier transform) or by increasing the sample concentration. However, second order effects can arise at these lower frequencies because of the smaller relative chemical shifts between the C4 and C5eq protons of the cyclotetrahydropyridine ring.

The strong coupling of these protons cause the spectrum of the C5ax proton to become "deceptively simple" (*27*) making any conformational analysis based on the value of J_{5ax-4} ambiguous. The second order effects can be eliminated by the preparation of the primary acid product in D_2O. This generates a nonspecific label at the C5 position, i.e., two diastereomers of the acid product, one with deuterium in the C5ax position and one with deuterium in the C5eq position. (Preparation of the primary acid product in D_2O has no effect on the absolute configuration at either the C4 position or the C6 position). Therefore, even at 60 MHz the spectrum for the C4, C5ax, and C6 protons of the C5eq-deuterated diastereometer will be first order. The loss of half the intensity of the C5ax proton absorption by the preparation of the primary acid product in D_2O is compensated for by the elimination of the large geminal coupling constant to the

C5eq proton. The spectrum of the C5ax proton will then simplify to a doublet of doublets with coupling contants of 9.5 Hz and ~13 Hz for a C4A proton and a B deuterium label; and a doublet of doublets, with coupling contants of 9.5 Hz and ~5.5 Hz for a C4B proton and an A deuterium.

The fact that the coenzyme analogs are enzymatically labeled with the identical configuration as NAD^+ indicates that differences in coenzyme conformation in solution, if any, probably do not affect the stereospecificity of the enzymatic reduction. In fact it appears that based on studies of the cyanide adducts of NAD^+ and $(AcPy)AD^+$ (*11*) both of these dinucleotides in solution have the same overall conformation with the B face of the pyridine ring against the adenine. The absolute assignment of the C4 protons of the dihydropyridine ring also yields important information about the conformation of NADH and the reduced analogs in solution. PMR studies of the C4 methylene proton of NADH (*11, 21*) have shown that, in the dinucleotide, the protons appear as a temperature-dependent, chemically nonequivalent A–B pattern[3]. The reduced analogs give a similar A–B pattern indicating that the relative position of the A and B protons are unchanged when compared to that of NADH (Table 2). These results are consistent with the existence of a strong similarity in the overall tertiary conformation for these dihydropyridine coenzymes in solution as well (*11*).

The results of the enzymatic labeling do not eliminate the possibility that these coenzyme analogs might alter the specificity of reduction of other dehydrogenases. Also, the possibility is not ruled out that other NAD^+ analogs might be reduced with different specificity. The results do show that "backwards" labeling apparently is not a general phenomenon. The stereospecificity of the enzymatic reduction of any of these three analogs can now be established by comparison with the reaction of the analog with lipoyl dehydrogenase. Finally, the absolute configuration of the enzymatic reduction of any other NAD^+ analog can be easily determined from the PMR spectrum of its primary acid product. This study emphasizes the unique ability of high-frequency PMR spectroscopy to investigate the stereochemistry of enzyme catalyzed reactions.

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Note added in proof. The stereospecificity of the reduction of (3-cyanopyridine)AD^+ has been determined by the Cornforth method to be identical to that of NAD^+. J. F. Biellmann, C. G. Hirth, M. J. Jung, N. Rosenburg, and A. D. Wrixon, *Eur. J. Biochem. 41*, 517 (1974).

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[3] Here, A–B stands for the NMR nomenclature for two strongly coupled nuclei, A and B where the coupling constant J_{AB} is approximately equal to or greater than the chemical shift difference between the two proton absorptions.

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