220 MHz Nuclear Magnetic Resonance Spectra of Oxidized and Reduced Pyridine Dinucleotides

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SUMMARY

1. The two \( C_1 \) protons of the dihydropyridine ring of reduced coenzymes appear as an AB spectrum in the 220 MHz NMR system. This indicates that they are chemically and magnetically nonequivalent.

2. When the oxidized coenzyme is converted to the reduced coenzyme, the coupling constant of \( C_1 \)H of ribose adjacent to the pyridine increased by about 3.5 Hz, whereas the same of the ribose adjacent to the adenine remained constant. This indicates that, when the oxidized pyridine dinucleotide is reduced, the ribose attached to the pyridine ring undergoes a conformational change, the conformation of the ribose attached to the adenine remaining the same in both oxidized and reduced coenzymes.

3. The \( C_1 \)H of the ribose adjacent to pyridine of reduced coenzyme is substantially broadened, and this broadening increased at elevated temperatures. This is due to onset of unresolved nitrogen spin-spin coupling as a result of the alteration of the relaxation time of nitrogen dominated by quadrupole coupling. This observation provides the most unequivocal method for assigning the chemical shift of the \( C_1 \)H of ribose attached to the pyridine of reduced coenzyme.

4. Starting changes take place in the shape and position of the ribose protons as a result of the reduction of the oxidized coenzyme. It is thought to result from an alteration in the geometric posture of the diphosphate backbone as a result of the reduction.

Nuclear magnetic resonance studies (1, 2) have strongly indicated that both oxidized and reduced pyridine dinucleotides (except their hypoxanthine analogues) exist in a folded conformation in which the adenine and pyridine rings are stacked in parallel planes. In a folded conformation, the two \( C_1 \) protons of the dihydropyridine ring of the reduced coenzyme are environmentally nonequivalent and hence would be expected to appear at different fields in their NMR spectra (see Fig. 4 in Reference 1). The 60 MHz spectra reported by Meyer, Mahler, and Baker (3) and Jardetzky and Wade-Jardetzky (2) and the 100 MHz spectra reported by Hollis (4) showed them to appear at the same field. The spectra of the \( geminal \) \( C_1 \)H protons of the dihydropyridine ring of DPNH and TPNH taken in the 220 MHz NMR system (5, 6) are shown in Fig. 1. The spectra are classic cases of AB spectra where the ratio of coupling constant (\( J \)) to the difference in chemical shift (\( \nu_\delta \)) is large and indicates that the two protons are magnetically nonequivalent. Theoretically calculated (7) geminal H-H spin-spin coupling constants as a function of the HCH bond angles vary from 32.3 Hz to 12.1 Hz as the HCH angle increases from 100° to 110°. The differences in chemical shifts between the two \( C_1 \) protons must be small, because the differences are in agreement with our observation that in the hypoxanthine analogue there is significantly less interaction between the base pairs (1, 9). The actual chemical shifts of the two \( C_1 \) protons were calculated (10) from the expression \[ C = \frac{1}{2}(\nu_\delta)^2 + J^2/2 \] where \( C \) = [difference in chemical shifts between Bands 2 and 3 (Fig. 1)] + \( J^2/2 \); \( J \) = coupling constants of the adenine ring and the other stereospecific to the A side, is not surprising in view of the nonequivalence of the electronic and geometrical environments of these two protons. The present observation of magnetic nonequivalence of the two \( C_1 \) protons may not be taken as an evidence for a stable puckered boat geometry for the dihydronicotinamide ring in which one proton is quasiequatorial and the other quasiequatorial. This is because the x-ray diffraction of the crystalline N-substituted 1,4-dihydronicotinamides (11) shows that the dihydronicotinamide ring is planar. The 100 MHz NMR spectrum of 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide supports the x-ray evidence (12). We believe that the folding of the adenine ring over the dihydropyridine ring (Fig. 4 of Reference 1) induces environmental nonequivalence on the two \( C_1 \) protons of the dihydropyridine ring of the dinucleotide. Such a concept agrees with the observation that the value of \( \nu_\delta \) decreases as one goes from TPNH or...
**AB Spectrum**

\[ \frac{J}{\nu_0} = \text{Large} \]

**TPNH**

Band No. 1 is Lost in Impurities

**DPNH**

Impurities

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**Fig. 1.** 220 MHz NMR spectra of the two C4 protons of the dihydro-pyridine ring of DPNH and TPNH. The total area covered by Bands 1, 2, 3, and 4 is equal to that of two protons. Downfield from the DPNH spectrum there is a set of small signals from unidentified impurities. In the case of TPNH, the spectrum of the C5 protons has moved downfield, and hence Band 1 is vitiated by the impurities whose resonance comes at the same field in both DPNH and TPNH.

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Theoretically, the reduction of the oxidized coenzyme should affect the chemical shifts of adenine and pyridine C1 protons as follows. (a) The dihydro-pyridine C1H of the reduced coenzyme should appear at a higher field than the pyridine C1H of oxidized coenzyme because as a result of the reduction, the full positive charge on pyridine N1 has been removed. The diminution of ring current when the ring is reduced would, however, affect the upward shift. (b) The dihydro-pyridine C1H of reduced coenzyme should appear at a higher field than the adenine C1H of reduced coenzyme because, unlike the dihydro-pyridine ring, the conjugated adenine could aid the polarization of the glycosidic linkage by delocalization. The Pullman (13) molecular orbital calculations for DPNH show that the net charge (which is a measure of the loss in an atom's electronic charge brought about by conjugations) for adenine N6 is 0.407 and for dihydro-pyridine N1 is 0.347. (c) The adenine C1H of the reduced coenzyme should appear at a lower field than the adenine C1H of the oxidized coenzyme because the ring current magnetic anisotropy of an aromatic pyridine ring is larger than that of a nonconjugated nonaromatic dihydro-pyridine ring. This is because the C1H of adenine of DPN lies in the diamagnetic shielding area of the pyridine ring current, as a comparison of the chemical shifts of adenosine diphasphoribose and DPN indicates (1). These three points could be reconciled only by assigning the downfield doublet (Fig. 2) to adenine C1H and the upfield doublet to dihydro-pyridine C1H in the case of the reduced pyridine dinucleotides (see below for unequivocal assignments).

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Strikingly in all the dinucleotides, when the oxidized coenzyme is converted to the reduced coenzyme, the C1H of ribose adjacent to pyridine coupling constant has increased by about 3.5 Hz (Table I). On the other hand, the adenine J23 values in the DPNH to NHDH, an observation compatible with the decreased intramolecular interaction between the base pairs in the hypoxanthine analogue (1, 9).

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**Fig. 2.** 220 MHz NMR spectra of pyridine C1H (PC1H) and adenine C1H (AC1H) of DPN and DPNH, and NHD and NHDH. The assignments in the oxidized coenzymes are according to Jardetzky and Wade-Jardetzky (2). Our assignments in the reduced coenzymes, discussed in the text, do not agree with those of Jardetsky and Wade-Jardetzky (2) who have made their assignments from a confused overlapping 60 MHz NMR spectrum. Note that in both oxidized and reduced coenzymes, the resonance from adenine C1H is sharp, but the pyridine C1H had substantially broadened and its coupling constant has increased in the reduced coenzyme.
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ribose protons C'2H, C'3H, C'4H, and C'5H2 and the adenine C'1H
as a result of the reduction of the oxidized coenzyme. The pyridine
a C'1-endo-C'2-exo conformation2 (14). In addition to the increase
in electronegativity brought about by reduction of DPN, because
such a change in electronegativity cannot affect the coupling
constants by any more than a few tenths of a cycle. Hence the
increase in J1,2 of pyridine ribose by about 3.5 Hz on the reduc-
tivity between the pyridine ring of DPN and the dihydropyridine
ring of DPNH, because such a factor has to affect the pyridine
and pyridine C'dH, C'sHz and adenine C'2H and C'5H2 protons.
This selective broadening of the dihydropyridine C'1H resonance
increased at elevated temperatures, indicating that N14 relaxa-
tion is responsible for this. Upon reduction, the electronic
properties of the pyridine ring, specifically, the local electronic
environment of the nitrogen atom, are altered. This would in
Fig. 3, top and bottom, shows the high frequency NMR spectra of
DPN and DPNH upfield of the HDO peak. Comparison of the
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TABLE I
Chemical shifts and coupling constants of certain pyridine and adenine
protons in pyridine dinucleotides

<table>
<thead>
<tr>
<th>Compounds</th>
<th>PC6Hs</th>
<th>PC6H</th>
<th>AC6H</th>
<th>AJ1/2</th>
<th>BJ1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN</td>
<td>Hs</td>
<td>Hs</td>
<td>Hs</td>
<td>1338.3</td>
<td>1314.3</td>
</tr>
<tr>
<td>DPNH</td>
<td>584.3</td>
<td>598.9</td>
<td>1332.1</td>
<td>1337.1</td>
<td>5.7</td>
</tr>
<tr>
<td>NHD</td>
<td>627.4</td>
<td>634.2</td>
<td>1336.0</td>
<td>1329.7</td>
<td>5.8</td>
</tr>
<tr>
<td>NHDH</td>
<td>585.9</td>
<td>599.9</td>
<td>1334.7</td>
<td>1348.6</td>
<td>5.3</td>
</tr>
<tr>
<td>TPN</td>
<td>602.7</td>
<td>620.7</td>
<td>1335.5</td>
<td>1321.5</td>
<td>5.1</td>
</tr>
<tr>
<td>TPNH</td>
<td>573.7</td>
<td>591.7</td>
<td>1339.1</td>
<td>1332.0</td>
<td>4.6</td>
</tr>
</tbody>
</table>

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change in the molecular geometry of the diphosphate backbone, a geometry different from that in the oxidized coenzyme, and that this geometric posture is such that it enables the P=O and the hydroxyl groups to shield the adenine $C_1^A$H and $C_2^A$H and the pyridine $C_1^P$H, $C_2^P$H, $C_3^P$H, and $C_4^P$H.

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Binding of Diffusible Molecules by Macromolecules: Rapid Measurement by Rate of Dialysis*

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SUMMARY

A rapid technique for the determination of the dissociation constant and the number of binding sites of an enzyme-substrate complex has been developed. The method is based on measuring the rate of dialysis of radioactive substrate from an enzyme-substrate equilibrium mixture. Accurate results can be obtained quickly, thus permitting studies with somewhat labile substances.

The standard procedure for the measurement of the binding of small molecules (ligands) by macromolecules is equilibrium dialysis. This procedure ordinarily requires several hours for attainment of diffusion equilibrium across a membrane, although in most cases the actual chemical equilibrium for the binding reaction can be reached within a fraction of a second. In the method to be described here, the equilibrium concentration of free ligand can be measured within 1 or 2 min after mixing the components, by determining the rate of dialysis of the radioactive ligand across a membrane. Since the rate measurement involves withdrawal of a negligible fraction of the total ligand, the same mixture can be used for a series of equilibrium measurements at increasing concentrations of unlabeled ligand. In this manner, all of the data for a Scatchard plot (1) to determine the number of binding sites on the macromolecule can be collected within 10 to 20 min from a single sample.

In the present work, the application of this technique to the binding of sugars by one of the yeast hexokinases is described. Preliminary accounts (2) of the results on binding of sugars and nucleotides by various yeast hexokinases have appeared elsewhere.

The apparatus (Fig. 1) consists of a dialysis cell with an upper chamber, containing the enzyme and labeled substrate, separated by a membrane from a lower chamber, through which buffer is pumped at a constant rate and from which the effluent is sampled for measurement of radioactivity.

Fig. 2 (curve with enzyme) illustrates the procedure used and the results obtained in a typical binding measurement. At the beginning of a determination, labeled substrate is introduced into the protein solution in the upper chamber, usually at a molarity considerably below that of the protein. Protein concentrations were in the range of 4 to 40 × 10⁻³ M in terms of total binding sites, in order to estimate the enzyme-substrate dissociation constants, ranging from 10⁻³ to 10⁻⁴ M, encountered with the hexokinase system. Specific radioactivities of approximately 150 mCi per mmole for ¹⁴C-glucose (from New England Nuclear) and 4 Ci per mmole for ³H-ATP (from Schwarz Biochemicals) gave satisfactory counts in the dialyze. The labeled substrate equilibrates immediately with the enzyme, and the rate at which radioisotope enters the lower chamber is proportional to the concentration of unbound labeled substrate in the upper chamber. This rate can be measured by determining the concentration of isotope in the effluent from the lower chamber. After a sufficient volume of buffer has passed to permit a steady state to be reached, i.e. when the rates for isotope entering and leaving the lower chamber are practically equal, the concentration in the effluent becomes a true measure of the concentration in the upper chamber.² The volume required to reach this steady state is


²Let N be the number of molecules of isotopically labeled substrate in the lower chamber at any time t after addition of substrate to the upper chamber. Then,

\[ \frac{dN}{dt} = S_1 \cdot D - N \frac{f}{V} \]

where \( S_1 \) is the concentration of free labeled molecules in the upper chamber, \( D \) is a constant which depends on the nature of the diffusing molecule as well as the properties of the dialyzing apparatus, \( f \) is the volume of buffer flowing through the lower chamber per unit of time, and \( V \) is the volume of the lower chamber. The first and second terms show, respectively, the rate of entrance and exit of isotope from the lower chamber. In the steady state (\( dN/dt = 0 \)), the concentration \( N/V \) of labeled molecules in the lower chamber becomes \( S_1 \cdot D/f \). The time course for approach to this steady state concentration is given by the integrated form of the above equation

\[ \frac{N(t)}{V} = \frac{S_1 \cdot D}{f} \left(1 - e^{-\left(\frac{t}{t(V)}\right)}\right) + \frac{N(0)}{V} e^{-\left(\frac{t}{t(V)}\right)} \]
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