PROTON RELAXATION STUDIES OF DIPHOSPHOPYRIDINE COENZYMES

by

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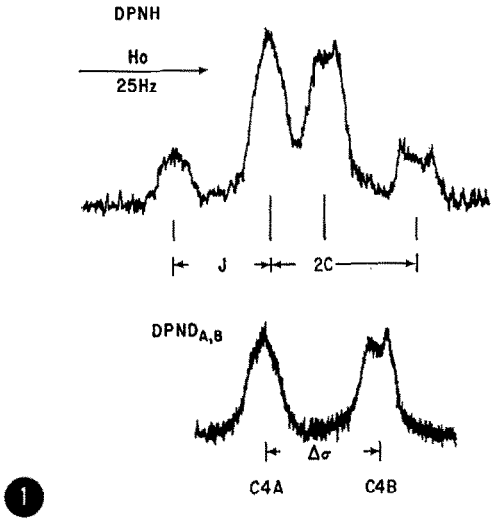
SUMMARY

The proton relaxation times of DPNH and 1-(2,6-dichlorobenzyl)-dihydronicotinamide (DCB-DHN) and their respective C4 deuterium-labeled dihydronicotinamide compounds were studied by Fourier transform NMR spectroscopy. The geminal C4 protons were found to exhibit unusually short relaxation times in comparison to the other dihydronicotinamide protons. Deuterium substitution at C4 increases the relaxation time of the remaining C4 proton. The deuterium relaxation time of the C4 deuterium labels has been measured. The small value of the deuterium T_1 precludes any significant contribution of the deuterium-proton scalar coupling. The advantages of C4 deuterium labeling in binding studies of DPNH with enzymes using NMR spectroscopy are also presented.

In DPNH, the C4 methylene protons protrude above and below the plane of the dihydronicotinamide ring. Since the absolute configuration of the C4A and C4B protons has been established (1) and their proton magnetic resonance absorptions assigned (2), the C4 protons should permit monitoring of the stereospecificity of interactions with dehydrogenases (3). However, attempts to obtain any information regarding specific interactions of the C4 methylene protons of DPNH with enzymes have been frustrated by the unusual broadness of the observed resonances (4).

Part of the broadness arises from the strong scalar coupling of the C4 protons. The large geminal coupling of -19 Hz combined with the small chemical shift nonequivalence of about 0.1 ppm produces a strongly coupled A-B pattern. As a result the C4 methylene proton resonances appear as an A-B quartet at 220 MHz (5,6) and a coalesced broad singlet at 100 MHz (7). The chemical shift nonequivalence ($\Delta\sigma$) of the absorptions of an A-B pattern is given by the relationship, $\Delta\sigma = [(2C)^2 - J^2]^{1/2}$ where J is the mutual coupling constant in Figure 1.



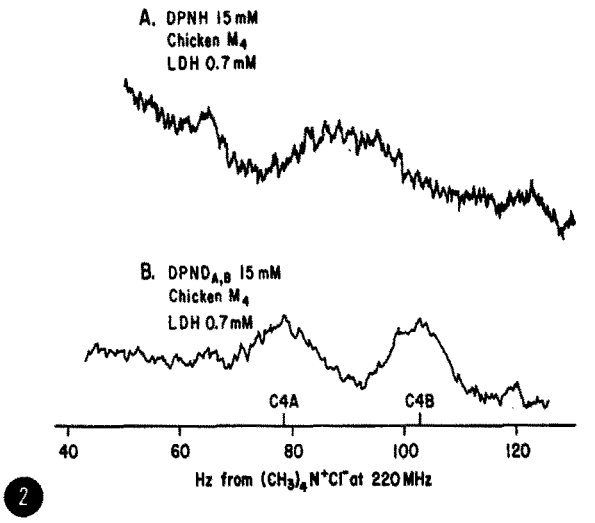


Figure 1. Comparisons of C4 proton resonance spectra of identical concentrations (50 mM) of DPNH AND DPND$_{A,B}$ at 22°C in neutral D_2O solution at 220 MHz where DPND$_{A,B}$ is a mixture of two C4-deuterium-labeled reduced pyridine dinucleotides.

Figure 2. Comparisons of the C4 proton resonance spectra (A) DPNH and DPND$_{A,B}$ in the presence of M_4 lactate dehydrogenase from chicken muscle at 220 MHz. (Coenzyme concentration ~15 mM, subunit concentration of enzyme ~0.7 mM, pD = 6.8, temperature 31°C).

The strong coupling makes accurate measurements of the chemical shift nonequivalence of the C4A and C4B protons difficult at 220 MHz and impossible at 100 MHz because of the inability to obtain accurate values of the parameter 2C. However, in addition to this effect, we have observed in the binding studies of DPNH with dehydrogenases (3) that the C4 protons are broadened to a much greater extent than either the C2 or C6 protons. This indicates that there is a difference in relaxation times of the C4 protons relative to that of the C2 and C6 proton. A strong dipole-dipole interaction between the C4 protons would be the most obvious cause of this difference. If true, then deuterium labeling of the C4 methylene should remove the proton-proton dipolar interaction making the relaxation properties of the remaining C4 proton more like those of the C2 and C6 protons, provided that the labeled-deuterium does not make any significant contribution to the geminal proton relaxation.

Longitudinal relaxation times (T_1) of the proton resonances in DPNH, $DPND_{A,B}$ and the dihydronicotinamide model compounds have been measured from the partially relaxed Fourier transfor spectra (PRFT) (9) where $DPND_{A,B}$ is a mixture of the two C4 deuterium-labeled dinucleotides prepared by the dithionite reduction of DPN^+ in D_2O (10). DCB-DHN and (4-deutero)-DCB-DHN have been prepared according to Kim and Chaykin (11). The results of the T_1 measurements are listed in Table I. As can be seen, the substitution of a deuterium at the C4 position in both DCB-DHN and DPNH substantially increases the relaxation time of the remaining C4 proton. As a result the T_1's of the C4 proton in (4-deutero)-DCB-DHN and $DPND_{A,B}$ are comparable to the T_1's of either the C2 or C6 protons.

These observations suggest that deuterium has little effect on the relaxation of the remaining C4 proton. To explain this phenomenon, further discussion of the relaxation mechanisms arising from deuterium is necessary. The deuterium nucleus posses a smaller gyromagnetic ratio than the proton, hence the effect of the deuterium-proton interaction on the proton relaxation can be no greater than 5% of that of the proton-proton dipolar interaction (12). In addition, the relaxation times of the C4 deuterium (T_{1D}) have been measured for the labeled compounds and the T_1 values are also listed in Table I. The relationship between the proton scalar relaxation and the deuterium relaxation is given in Equation 1.

$$\frac{1}{T_{1H}} = \frac{8\pi^2}{3} J^2(S+1)S \frac{T_{1D}}{1 + (\omega_H - \omega_D)^2 T_{1D}^2} \qquad (1)$$

where J is the proton-deuterium coupling constant (equal to about one-sixth of the proton-proton coupling constant of -19 Hz); S = 1 is the nuclear spin of deuterium, and ω_H and ω_D are the resonance frequencies of the proton and deuterium, respectively.

The dipolar coupling of the C4 geminal protons of DPNH results in a shorter T_2, hence broader lines for the C4 protons in comparison to the other protons. For DPNH in solution $T_1 \geq T_2$, thus from our measurements of T_1 (Table I), there

TABLE I. Proton and Deuterium Longitudinal Relaxation Times (T_1) of the Dihydronicotinamide Ring of DCB-DHN, (4-Deutero)-DCB-DHN, DPNH and $DPND_{A,B}$.

	Protons T_1's				Deuterium T_1's		
Compound	C2H	C6H	C5H	C4H	C4D	Q_D[e]	τ_c[f]
DCB-DHN[a]	3.6	2.6	2.3	0.9			
(4-Deutero)-DCB-DHN[a]	3.5	2.5	2.9	3.9	0.042[c]	165 KHz	
DPNH[b]	0.37	0.40	-	0.13			
$DPND_{A,B}$[b]	0.38	0.40	-	0.42	0.0085[d]		3×10^{-10}

The T_1 values (in seconds) are accurate to within 10%.

[a] 0.1 M in 80% $CDCl_3$/20% CD_3OD at 16°C.

[b] 0.05 M in D_2O at 16°C.

[c] 0.2 M in 80% $CHCl_3$/20% CH_3OH at 16°C.

[d] 0.2 M in H_2O at 16°C.

[e] Q_D: Deuterium quadrupolar coupling constant.

[f] τ_c: Correlation time of geminal C4 protons in seconds at 16°C.

should be over a three fold difference in the observed linewidth of the C4 protons and the other protons. However, this difference is difficult to detect because it is masked by the extensive, unresolved, scalar coupling of all the protons and the contribution to the line width of magnetic field inhomogeneity.

Formation of a DPNH-dehydrogenase complex results in the immobilization of the coenzyme, increasing τ_c for DPNH by 2-3 orders of magnitude. The long correlation time causes T_2 to be short, hence the proton lines are expected to be broad (100-140 Hz). In our enzyme binding study, DPNH is in intermediate to fast exchange, thus the observed line width (T_2) is the weighted average of the linewidths in the free and bound state. The decrease in T_2 is expected to be uniform (to a first approximation), hence the difference in the line widths of the C4 and the other protons should become quite apparent. We observe that the C4 proton absorptions are broadened very severely in comparison to the other protons (4), so severely in fact that no useful information can be obtained on the chemical shifts of the C4 protons induced by the enzyme.

The extra broadening (shorter T_2) of the C4 protons arising from the gemi-

nal, dipolar coupling is eliminated in $DPND_{A,B}$. Because $\tau_c\omega_o >> 1$, the contribution from geminal-deuterium scalar coupling is negligible. As a result, the T_2 of the remaining C4 proton of $DPND_{A,B}$ in the enzyme bound state is comparable to the other protons and as we have observed the line widths are all nearly equal.

Since the condition that $(\omega_H - \omega_D)T_{1D} >> 1$ holds for both the model compound and the dinucleotide, the contribution of the deuterium scalar coupling to the proton T_1 is negligibly small. Furthermore, the short deuterium relaxation times observed in these compounds also mean that $1/2\pi J >> T_{1D}$. Thus the protons are effectively decoupled from the deuterium (12). Instead of observing a triplet pattern with a 3 Hz coupling constant, only broad singlets are observed in Figure 1 for the remaining C4 protons; there is no indication of any deuterium-proton scalar coupling. The broadness of the C4 protons in DPNH and $DPND_{A,B}$ is due to long range scalar coupling to the C2, C5 and C6 protons and is comparable for the two dinucleotides.

Knowing the proton and deuterium relaxation times of DPNH and $DPND_{A,B}$ as well as the inter-proton distance between the geminal C4 protons, the deuterium quadrupolar coupling constant, Q_D, and the correlation time, τ_c, can be estimated as shown in Table I. The calculated Q_D is comparable to other similar deuterium labeled compounds (13) and is in good agreement with the value of Q_D that can be calculated for DCB-DHN from measurements of the ^{13}C relaxation time of C4 (Table I).

Besides increasing the relaxation time of the C4 protons, the introduction of deuterium has another greater advantage for use in enzyme binding studies. At 220 MHz the central peaks of the C4 methylene A-B quartet of DPNH nearly overlap and will coalesce upon relatively small increases in the peak width as shown in Figure 2. This precludes obtaining any information regarding the specific chemical shift changes of either the C4A and C4B proton upon binding to dehydrogenases. Deuterium labeling eliminates the strong geminal coupling, allowing the full chemical shift nonequivalence of the C4A and C4B protons to be observed

in the enzyme bound state (compare the striking differences between the two spectra of C4 proton resonances in Figure 2) (3). At 100 MHz the methylene protons of DPNH are completely coalesced into a broad singlet even though they are 10 Hz nonequivalent. When nonspecific or specific deuterium-labeled dinucleotides are used at 100 MHz, one can now detect the chemical shift non-equivalence of the C4 protons. With the advantages of using C4 deuterium-labeled coenzymes, we have been able to show that C4A proton resonance of reduced coenzyme shifts downfield by 0.5 ppm, but C4B proton resonance remains unchanged upon binding to lactate dehydrogenase from chicken muscle (3). Further studies regarding the detailed explanations of these differential shifts of C4 proton resonances of DPNH are now in progress.

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