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Hydrogen bonding effects on ^{31}P NMR shielding in the pyrophosphate group of NADPH bound to *L. casei* dihydrofolate reductase

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A comparison of ^{31}P NMR chemical shift data and X-ray structural data [Filman, D.J., Bolin, J.T., Matthews, D.A. and Kraut, J. (1982) J. Biol. Chem. 257, 13663–13672] for complexes of NADPH with *L. casei* dihydrofolate reductase indicates that solvation effects play a major role in influencing the ^{31}P shielding of the pyrophosphate nuclei whereas changes in P-O-C_5-H_5' torsion angle have little effect.

^{31}P NMR; NADPH; DHFR; Solvation

1. INTRODUCTION

The mode of binding of NADPH to the enzyme dihydrofolate reductase (DHFR) has been well-characterised using X-ray and NMR methods [1]. Extensive ^{31}P NMR studies of the DHFR·NADPH and DHFR·NADPH·MTX of the *L. casei* enzyme have shown that whereas the ^{31}P resonance of the nicotinamide pyrophosphate P(n) is shifted only slightly on binding (high frequency shift 0.16 to 0.15 ppm), the ^{31}P resonance of the adenine pyrophosphate P(a) shows a large low frequency shift (2.69–2.55 ppm) [2–5]. It was suggested that this shift might arise from a combination of electric field effects and charge redistribution effects accompanied by small changes in the P-O-P bond angles [2]. Based on Gorenstein's empirical correlation of ^{31}P chemical shifts with O-P-O bond angles [6] it is evident that quite minor changes in angle (<1°) could give rise to the chemical shift variations seen in these complexes. Such changes in O-P-O bond angles could result from differences in interactions between the pyrophosphate oxygen atoms and the neighbouring protein residues in the different complexes [3]. Gorenstein and collaborators [7–10] have further suggested that the ^{31}P chemical shifts are also dependent on P-O-C-H torsional angles and that P-O-P bond angle and P-O-C-H torsion angle effects are in fact related phenomena. Molecular orbital calculations for phosphate diester groups have indicated that rotation about the P-O-C-H torsion angle could produce O-P-O bond angle changes and some support for this prediction was provided by X-ray structural data from some nucleotides with large differences in torsion angle [11,12]. In the NMR studies of the DHFR·NADPH complexes, measurements of $^3J_{P\text{-}O\text{-}C\text{-}H}$ coupling constants allowed the P-O-C_5-H_5' torsion angles to be estimated. From the coupling constants it was found that for the P(n) group a 50° change in the torsion angle accompanied the binding although its ^{31}P signal was relatively unshifted. In contrast the corresponding torsion angle for the P(a) group did not change on binding even though its ^{31}P signal shows a large low frequency shift [2]. Clearly the factors controlling the ^{31}P shielding are still poorly understood. The change in this torsion angle in the P(n) group was subsequently also observed in the crystallographic studies on the DHFR·NADPH·MTX complex [13–16].

In this communication, we draw attention to the effects of changes in hydrogen bonding on the ^{31}P nuclear shielding of the two pyrophosphate phosphorus nuclei of NADPH in its complex with *L. casei* DHFR as deduced from the striking differences in hydrogen bonding patterns of the two phosphate groups determined by X-ray crystallography [15,16]. These factors have received relatively little attention in interpreting ^{31}P chemical shifts in biomolecules.

2. RESULTS AND DISCUSSION

Crystallographic refinement of the ternary complex *L. casei* DHFR·NADPH·MTX [15,16] has revealed that the pyrophosphate oxygens are extensively hydrogen bonded and these findings are summarized in Table I. The oxygen O1P(n) of the nicotinamide 5' phosphate group is at a short hydrogen bonding distance (2.7 Å) from the oxygen of the water molecule 276, Wat-276, and forms two further hydrogen bonds with enzyme residues (peptide NHs of Ala-100 and Gln-101). The oxygen O2P(n) forms hydrogen bonds with two molecu-

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Table I

Hydrogen bonds between the pyrophosphate group of NADPH, bound molecules of H_2O and amino acid residues of *L. casei* DHFR (from reference [16])

Component of NADPH				Group in DHFR			Group in NADPH	
Group	Atom	Water molecules	Distance[a] (Å)	Residue	Atom	Distance[a] (Å)	Atom	Distance[a] (Å)
NMN ribose 5'-phosphate	O1P(n)	276	2.7					
	O1P(n)			Gln-101	Peptide N	2.9		
	O1P(n)			Ala-100	Peptide N	3.2		
	O1P(n)						O1A	3.1
	O2P(n)	302	2.7					
	O2P(n)	301	3.0					
	O2P(n)	276	3.5					
	O2P(n)			Arg-44	Peptide NH1	3.1		
AMN ribose 5'-phosphate	O1P(a)			Gly-99	Peptide N	3.1		
	O1P(a)			Ile-102	Peptide N	3.3		
	O1P(a)			Gln-101	Peptide N	3.4		
	O1P(a)						O1N	3.1
	O2P(a)			Thr-45	Oγ1	2.6		
	O2P(a)			Gly-99	Peptide N	3.0		
	O2P(a)			Thr-45	Peptide N	3.2		

The abbreviations (a) and (n) are used to denote atoms contained in adenine and nicotinamide mononucleotides.
[a]Distances are taken from the X-ray crystal structure [16].

les of H_2O, Wat-301 and Wat-302, and forms a further hydrogen bond to NH1 of Arg-44. Interestingly there are several molecules of water which are not at classical hydrogen bond distances from O1P(n) and O2P(n), but are hydrogen bonded to the directly bound molecules of water (Table II). This denotes further solvent accessibility of the bound solvation molecules of water. It can therefore be concluded that the solvent accessibility of the nicotinamide 5' phosphate group results in a largely aqueous-like environment similar to that of the free NADPH and therefore giving rise to similar ^{31}P NMR chemical shifts.

By contrast the solvent accessibility of the adenine 5' phosphate group is restricted and no interacting solvation molecules of water are observed in the X-ray structure. The oxygens of the adenine 5' phosphate, O1P(a) and O2P(a) are both only hydrogen bonded to protein residues (Table I).

Lerner and Kerns [17] have previously drawn attention to the possible effects of hydrogen bonding interactions on the ^{31}P NMR chemical shifts. These workers have reported detailed studies of solvent effects in a variety of cyclic nucleotides where the conformational state of the phosphodiester group is expected to be relatively fixed [18] thus reducing the possibility of solvent induced conformational changes, which might complicate the interpretation of the ^{31}P chemical shift. It was found that changes in solvation caused by the various organic solvents induce shifts to low frequency which can be greater than 3 ppm. The order of the low frequency shifts correlates with the hydrogen bond donating ability of the solvents indicating that hydrogen bonding to the phosphate group is a key factor in determining the magnitude of the solvent effects. It is interesting to note that all organic solvents used caused low frequency ^{31}P shifts relative to those measured in H_2O. They concluded that any uncharged organic solvent molecule would produce low frequency shifts of the ^{31}P NMR resonances relative to those observed in H_2O. It is thus reasonable to expect that the formation of hydrogen bonds of the adenine pyrophosphate group oxygens with the NH groups of protein residues would induce low frequency ^{31}P shifts compared to those in the nico-

Table II

Oxygen–oxygen distances between water molecules solvating the nicotinamide phosphate P(n)

	Water molecule	O···O Distance[a] (Å)
Wat-276	514	2.8
	301	2.9
Wat-301	276	2.9
	344	3.0
	302	3.2
Wat-302	516	2.7
	208	3.0
	301	3.2

[a]Distances are taken from X-ray structure [16]

tinamide pyrophosphate group which is exposed to solvation with molecules of H_2O. This is in excellent agreement with the observed direction and magnitude of the ^{31}P NMR pyrophosphate chemical shifts of the bound NADPH in the DHFR·NADPH·MTX complex compared to those of the free NADPH is aqueous solution. These changes in hydrogen bonding interactions could also result in O-P-O bond angle changes. However, it appears that changes in the P-O-C_5H_5' torsion angles are not important in contributing to the shielding of these nuclei. The finding that the torsion angle difference of 50° for the P(n) group in bound and free NADPH is not accompanied by a substantial change in ^{31}P shielding indicates that torsion angle effects are unimportant over this range.

The above observations thus suggest that the ^{31}P chemical shifts of the pyrophosphate phosphorus nuclei observed for NADPH bound to *L. casei* DHFR depend largely on a combination of hydration accessibility and hydrogen bonding interactions with changes in torsional angles playing an insignificant role.

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