CONFORMATIONAL CHANGES, DYNAMICS AND ASSIGNMENTS IN ^{1}H NMR STUDIES OF PROTEINS USING RING CURRENT CALCULATIONS

Hen egg white lysozyme

Stephen J. PERKINS*, Louise N. JOHNSON and David C. PHILLIPS
Laboratory of Molecular Biophysics, Zoology Department, South Parks Road, Oxford OX1 3PS

and

Raymond A. DWEK
Department of Biochemistry, South Parks Road, Oxford OX1 3QU, England

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1. Introduction

The prediction of the ^{1}H NMR spectra of proteins from the crystal structure is important for the understanding of the protein ^{1}H NMR spectrum. The assignment of the resolved ^{1}H NMR signals of the protein to specific residues in the primary sequence is complex and often ambiguous. Comparison between observed and predicted spectra in cases where the crystal structure of the protein is known should facilitate assignment and furthermore allow comparison of the crystal and solution conformations of the protein.

Two earlier studies using the Johnson-Bovey equation for ring current shifts for a benzene ring compared qualitatively the upfield ^{1}H NMR spectrum of lysozyme [1] and α-lactaalbumin [2] with the predicted spectrum respectively from the wire-model and regularized coordinates from the crystal structure of lysozyme. It was thus inferred that the conformation of these proteins in solutions is similar to lysozyme in the crystal structure.

This present study describes ring current calculations for the downfield (aromatic proton) spectrum of lysozyme based on the coordinates of the protein after real space refinement [3,4] and the Johnson-Bovey equation applied to aromatic amino acids [5–7]. Thus shift corrections to the ^{1}H chemical shifts of the free amino acids are calculated, which take into account the tertiary structure of the protein. To illustrate the potential of these calculations, they are used.

i. To assign four aromatic spin-coupled signals in the ^{1}H NMR spectrum of lysozyme to Trp 28.
ii. To estimate a lower limit for the motional 'flipping' dynamics of the tyrosine aromatic rings of lysozyme.
iii. To rationalise the chemical shift changes in the ^{1}H NMR spectrum on the binding of ligands to lysozyme.

A detailed description of the calculations for the whole protein will be presented in a subsequent paper.

Abbreviations: ^{1}H NMR, proton nuclear magentic resonance; β Me GlcNAc, β methyl *N*-acetyl-D-glucosaminoside; Trp, tryptophan; His, histidine; Phe, phenylalanine; Tyr, tyrosine

* Present address: Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8093 Zürich-Hönggerberg, Switzerland

2. Experimental

A FORTRAN program RCCAL was written to calculate the proton positions from the real space refined RS5D set of native lysozyme coordinates [4],

Table 1
Parameters used in the Johnson-Bovey equation

		Six membered ring	Five membered ring
1.	Ring radius a	0.139 nm	0.1182 nm
2.	Separation of current loop from ring plane q	0.459 ring radii	0.5398 ring radii
3.	Ring current factor i [7]		
	His		0.53
	Tyr	0.94	
	Phe	1.00	
	Trp	1.04	0.56
4.	Carbon–hydrogen bond length of aromatic protons 0.1084 nm		
	Protein coordinates:	Lysozyme RS5D set [4] lysozyme-β Me Glc NAc real space refined [14]	

using standard subroutines [8] and bond lengths, and to calculate the mean square planes of the aromatic rings of lysozyme [9]. Using these, RCCAL calculated the ring current shifts using the parameters summarised in table 1 in the Johnson-Bovey equation [5]:

$$\Delta\delta \times 10^{-6} = i\frac{ne^2}{6\pi mc^2 a}\frac{1}{((1+\rho)^2+z^2)^{\frac{1}{2}}}\left[K+\left(\frac{1-\rho^2-z^2}{(1-\rho)^2+z^2}\right)E\right]$$

in e.s.u. units where $\Delta\delta$ is the ring current shift in ppm; n is the number of electron; a is the ring radius; e,m,c are the standard constants; i is the Geissner-Prettre-Pullman ring current factor [7]; K,E are the first and second complete elliptic integrals and are a function of ρ,z and q where q is the separation of the aromatic ring from the π-electron cloud and ρ and z are the radial and elevational cylindrical coordinates respectively (all three being in units of ring radii a).

It is known that shielding effects arising from carboxyl, amino, sulphide, hydroxide and peptide groups may influence proton chemical shifts [10]. In this connection the non-polar environment of the C(4)H, C(5)H, C(6)H and C(7)H aromatic protons of Trp 28 compared to the other aromatic protons is particularly striking. The nearest sidechain polar atom is the sulphur of Met 105, which is 0.5 nm from each of these four protons. Further no peptide carbons are within 0.4 nm and no ionisable groups are within 1.0 nm of the four protons. Accordingly the calculations for the four Trp 28 protons are more reliable than would otherwise be the case.

3. Results and discussion

3.1. *Control ring current calculations*

The predicted ring current shifts for the 65 aromatic protons of lysozyme are reported in table 2. To investigate the performance of the Johnson-Bovey equation, the calculations were repeated varying the four parameters of table 1. Increase of the ring radii by 25% produced up to 50% increases in the calculated shifts, but 25% changes in q had an almost negligible influence. Resetting all the rings as phenyl rings or increasing the C–H bond lengths by 25% produced variable increases of up to 0.1 ppm. In all these calculations, the predicted shifts for the aromatic protons were found to remain in the same relative order of values; this is significant for Trp 28 and Trp 63 where the interaction of the 'cone' of zero ring current shift is evident (table 2). Thus, while there may be some uncertainties in the magnitudes calculated, the use of relative values of ratios of ring current shifts is expected to be more effective. For aromatic protons in particular, the advantage of the constraints inherent in the use of 4

Table 2

Aromatic residue	Protons and predicted shifts (in ppm)[a] for the lysozyme RS5D coordinates					
His 15		C(2)H 0.02	C(4)H 0.09			
	C(2,6)H	C(3,5)H		C(3,5)H	C(2,6)H	
Tyr 20	− 0.04	− 0.03		− 0.09	− 0.26	
Tyr 23	0.09	− 0.03		0.08	0.01	
Tyr 53	− 0.03	− 0.01		− 0.04	− 0.07	
	C(3,5)H	C(3,5)H	C(4)H	C(3,5)H	C(2,6)H	
Phe 3	0.09	0.04	0.03	0.01	− 0.01	
Phe 34	0.07	− 0.30	− 0.26	− 0.06	0.05	
Phe 38	0.10	0.23	0.23	− 0.04	− 0.01	
	C(2)H	N(1)H	C(4)H	C(5)H	C(6)H	C(7)H
Trp 28	− 0.03	− 0.15	0.47	0.71	0.102	− 0.16
Trp 62	0.04	0.03	0.29	0.10	0.04	0.05
Trp 63	− 0.09	0.13	− 0.04	− 0.01	0.08	0.24
Trp 108	− 0.02	− 0.01	0.37	0.29	0.01	0.07
Trp 111	0.16	0.06	0.00	− 0.04	− 0.15	− 0.16
Trp 123	− 0.08	− 0.15	− 0.10	− 0.01	0.22	− 0.22

[a] The ring current shift of the own aromatic ring is subtracted from the values given here and in table 3. Negative values symbolize downfield shift changes from the three amino acid chemical shifts

Chemical shift perturbations of the strongly ring current shifted tryptophan [11] from the shifts in the amino acid[b]

C(4)H	C(5)H	C(6)H	C(7)
0.9	0.9	0.4	− 0.4
− 0.2	0.3	1.0	0.7

[b] Two values are given as it is not known ab initio from the NMR studies which signal is the C(4)H or C(7)H proton and which is the C(5)H or C(6)H proton. The crystal structure resolves the ambiguity as above. Lysozyme shifts: δ = 6.76 (doublet), 6.28 (triplet), 6.85 (triplet), 7.85 (doublet); spin coupled in that sequence [11]. Free Trp signals: C(4)H 7.7; C(5)H 7.2; C(6)H 7.3; C(7)H 7.5 [16]

or 5 distinct locii in a rigid frame (i.e., the spin coupled protons of Trp, Phe and Tyr) relative to the main perturbing centre is apparent.

3.2. *Assignment of Trp 28 resonances*

In the spectrum of lysozyme, the strongly shifted aromatic resonances of a tryptophan residue have been identified by spin-decoupling (table 2, [11]). These were tentatively assigned to Trp 63 on the basis of an examination of the X-ray structure [12] and assuming that Trp 63 could interact significantly with the ring current fields from Trp 62. The real space refined coordinates of lysozyme show that this is unlikely since the centres of the two six-membered rings are 0.75 nm apart. At these distances, the ring current effects from Trp 62 are too small to cause significant shifts in the resonance positions of Trp 63 (and vice versa). This is also the case in the crystal structure of the lysozyme β Me GlcNAc complex [14]. Here the aromatic rings of Trp 62 and Trp 63 are defined in position by reason of the hydrogen bonds between the N(1)H protons and the O(6) and O(3) atoms, respectively, of the sugar. Even in this case the centres of the six-membered rings are 0.73 nm apart.

Bearing in mind the qualitatively satisfactory working of the Johnson-Bovey equation [1,2,12], inspection of table 2 suggests that the resonances tentatively assigned to Trp 63 should now be assigned to Trp 28. Not only are the absolute magnitudes of the shifts in better agreement, but the sequence of the ring current shifts is now well-predicted. The magnitudes of the four observed shift changes are each underestimated by 0.2–0.4 ppm in the calculations, in reasonable accordance with experience of the Johnson-Bovey equation [15]. The main source of the shifts on the Trp 28 protons is from the interactions with Trp 108, which is close to the C(5)H proton of Trp 28.

3.3. *Tryptophan N(1)H and C(2)H signals*

Six Trp C(2)H signals have been identified at δ = 7.63, 7.55, 7.30, 7.18, 7.08 and 7.03, with the Trp 108 C(2)H at δ = 7.08 [13]. The range of shifts is thus about ± 0.3 ppm from the free Trp C(2)H signal at 7.3 ppm [16]. The present calculations (table 2) suggest a range of + 0.16 to −0.09 ppm in ring current shift for these protons, which is in satisfactory agreement with the observed values.

Five of the six N(1)H signals of the Trp indole rings have been observed at δ = 10.73, 10.39, 10.26, 10.06 and 10.04, of which the signal at δ = 10.04 has been assigned to Trp 108 [13]. In contrast to the behaviour observed with the adjacent C(2)H protons, some of these are downfield shifted from the free Trp N(1)H signal at δ = 10.25 [17] by as much as 0.5 ppm. Such large downfield shifts are not predicted in table 2, and ring current calculations for the polar N–H protons thus appear less reliable than those for the non-polar C–H protons. It is likely that the chemical shifts of the Trp N–H protons in protein ^{1}H NMR spectra may be of use as a probe of the chemical environment of these N–H protons (i.e., the existence of and nature of hydrogen bonds involving these protons).

3.4. *Tyrosine signals*

All six tyrosine 2-proton doublets have been identified at 270 MHz [18] at δ = 6.71, 6.83, 6.98, 7.05, 7.09, 7.24. The range of thse shifts compared to free tyrosine where δ = 6.9 and 7.2 [16] are in accordance with the predicted low perturbations of the tyrosine signals through ring current effects (table 2).

Given the non-equivalence of the chemical shifts of the C(2,6)H and C(3,5)H protons of Tyr and Phe aromatic rings in the protein ^{1}H NMR spectrum, relatively slow motion on the NMR time-scale about the Cβ–Cγ axis gives rise to two one-proton signals in place of a single two-proton signal [19.20]. In such situations, the dynamics of this motion may be investigated [21–23]. For proteins such as lysozyme, only the fast exchange situation has been observed for the six tyrosine doublets and no easy analysis is possible. However multiplication of the chemical shift difference between the two C(2,6)H or C(3,5)H protons (table 2) by the spectrometer frequency gives directly an estimate of a lower limit on these 'flipping' rates of around 10^2 s^{-1}.

3.5. *Shift changes upon the binding of ligands*

A further application of these calculations is to interpret changes in the ^{1}H NMR spectrum of a protein that follows the binding of ligands or chemical modifications. In the crystal structure of lysozyme-β Me GlcNAc, a number of small conformational changes have been observed and analysed by real space refinement [14]; these include some of the aromatic rings. For example, the centre of the six-membered ring of Trp 108 moves 0.03 nm nearer to the C(5)H proton of Trp 28. The ring current calculation for the real space refined coordinates of lysozyme β Me GlcNAc (table 3) shows that the C(5)H proton of Trp 28 is shifted upfield by 0.2 ppm from that shift calculated for the native RS5D coordinates (table 2). On running the NMR spectra of lysozyme-GlcNAc and lysozyme-$(GlcNAc)_3$, the Trp triplet at δ = 6.28 ppm is observed to shift upfield by up to 0.2 ppm [24]. Thus there is a good agreement between the observed and calculated behaviour in the solution and crystal states.

For this kind of application to be productive, the ^{1}H NMR signal in question has to be influenced by a large ring current shift initially. A change of 0.1 ppm for such a large shifted signal is easily observed in ^{1}H NMR spectra and indicates a movement of about 0.1 nm in the protein if it cuts across the isoshielding lines of ring current shifts; an equivalent or higher precision than the present accuracy of highly refined protein structures [25,26]. On the other

Table 3

Aromatic residue	Protons and predicted shifts (in ppm) for the lysozyme-β Me Glc NAc coordinates					
		C(2)H	C(4)H			
His 15		0.01	0.08			
	C(2,6)H	C(3,5)H		C(3,5)H	C(2,6)H	
Tyr 20	− 0.05	− 0.03		− 0.08	− 0.23	
Tyr 23	0.14	0.05		0.07	− 0.02	
Tyr 53	− 0.03	0.00		− 0.03	− 0.06	
	C(2,6)H	C(3,5)H	C(4)H	C(3,5)H	C(2,6)H	
Phe 3	0.09	0.04	0.04	0.02	0.00	
Phe 34	0.09	− 0.31	− 0.26	− 0.06	0.04	
Phe 38	0.11	0.23	0.17	− 0.04	− 0.01	
	C(2)H	N(1)H	C(4)H	C(5)H	C(6)H	C(7)H
Trp 28	− 0.02	− 0.16	0.52	0.91	0.03	− 0.12
Trp 62	0.12	0.07	0.36	0.12	0.06	0.07
Trp 63	− 0.06	0.15	− 0.05	− 0.03	0.06	0.21
Trp 108	− 0.03	− 0.01	0.35	0.24	− 0.04	0.04
Trp 111	0.16	0.05	− 0.01	− 0.05	− 0.15	− 0.17
Trp 123	− 0.08	− 0.15	− 0.10	0.01	0.31	− 0.19

hand, ring current effects only report on localised movements because of the approximately inverse-cubed dependence of ring current shift on distance. Thus while in the crystal structure the indole rings of Trp 62, Trp 63 and Trp 108 are all observed to move on addition of the inhibitor sugar, this particular shift change above has only resulted from the small movement of the Trp 108 ring relative to the C(5)H proton of Trp 28. This shows how NMR and X-ray studies can be used to complement each other.

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