

## MAPPING OF THE BINDING SITE OF *N*-FLUOROACETYL-D-GLUCOSAMINE AND ANALOGUES IN HEN EGG LYSOZYME BY $^1\text{H}$ AND $^{19}\text{F}$ -NMR TECHNIQUES WITH $\text{Gd(III)}$ AND $\text{Mn(II)}$ AS PARAMAGNETIC PROBES

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The  $^{19}\text{F}$ -NMR spectra of methyl 2-deoxy-2-fluoroacetamido  $\alpha$ - and  $\beta$ -D-glucosides (in 100%  $\text{D}_2\text{O}$ ) consists of triplets ( $\sim 225$  ppm upfield from  $\text{CFCl}_3$ ;  $J = 47$  Hz) separated by 0.46 ppm. The solutions were at (nominal) pH 5 in 0.1 M-imidazole- $\text{H}_2\text{SO}_4$  buffer. Under these conditions full deuteration of the sugar imido group can be assumed. In 25%  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$ , further splitting of the spectrum of pure methyl 2-deoxy-2-fluoroacetamido- $\beta$ -D-glucoside was observed. The two sets of triplets present, separated by 0.1 ppm, were attributed to the isotopic effect of -NH- and -ND- forms of the glycoside. Similar distinction between  $\alpha$ - and  $\beta$ -anomers of *N*-trifluoroacetyl-D-glucosamine in aqueous solution has been also observed two resonances [1,2] at  $-11.2$  and  $-10.9$  ppm (down-field from trifluoroacetone as internal reference) being present. These results substantiate the relative magnitude and marked environment dependence exhibited by fluorine, which renders certain fluorocompounds eminently suitable as NMR probes.

In the presence of lysozyme (3 mM), buffered aqueous solutions (pH 5,  $22^\circ$ ) of methyl *N*-fluoroacetyl- $\beta$ -D-glucosaminide show pronounced down-field changes in  $^{19}\text{F}$ -chemical shifts [3,4], in contrast to the  $\alpha$ -analogue where the effect is relatively slight. Plots of the monosaccharide-concentration dependence (0.1–0.2 M) of the shifts (at constant enzyme concentration) give a value of  $40 \pm 10$  mM for the dissociation constant ( $K_S$ ) of methyl *N*-fluoroacetyl- $\beta$ -D-glucosaminide and  $-1.5 \pm 0.2$  ppm for the shift difference ( $\Delta$ ) of the free and bound forms of the fluorosugar (corresponding to the chemical shift of the fully-saturated monosaccharide–enzyme complex). Values of  $\Delta$  have been determined for other

*N*-fluoroacetyl- and *N*-trifluoroacetyl-glucosamine derivatives (Butchard et al. 1972)[5].

From X-ray diffraction data (C.C.F. Blake and M.A. Rabstein, unpublished results) it is known that  $\text{Gd(III)}$  binds to lysozyme at a strategic position between Asp 52 and Glu 35. This enzyme-bound cation, acting as a paramagnetic probe, is found to broaden the  $^1\text{H}$  and  $^{19}\text{F}$ -resonances of a fluorosugar. Under conditions of fast exchange of the fluorosugar between free solution and the enzyme-bound form, the broadening is a function of the distance between the  $\text{Gd(III)}$  and the nucleus under observation [6,7] and of a correlation time which can be estimated from the solvent water–proton relaxation rates in the system. A detailed treatment of the theory relating  $^{19}\text{F}$ -linewidths to the fluorine-metal distances together with conditions for its applicability has been published [8]. Errors arising from the use of water-relaxation time to calculate rotational correlation time have been found to be small and to be within the accumulated experimental errors quoted for the distances found between the metal ion (Gd or Mn) and H or F. The data for line broadening under various conditions is given in the table. The distance was calculated to be  $0.55 \pm 0.02$  nm between  $\text{Gd(III)}$  and the F atom in *N*-fluoroacetyl- $\alpha$ -D-glucosamine (and its methyl glycoside) and  $0.51 \pm 0.02$  nm for the corresponding  $\beta$ -analogues. This distance is somewhat shorter than those evaluated from the molecular model of lysozyme, with the sugars positioned according to the high resolution co-ordinates of  $\alpha$ - and of  $\beta$ -N-acetylglucosamine, ( $0.60 \pm 0.08$  and  $0.73 \pm 0.08$  nm, (accumulated

experimental error)) suggesting that there may be a slightly different orientation of the fluorinated sugars. With the  $\alpha$ - and  $\beta$ -isomers of *N*-trifluoroacetyl-D-glucosamine the Gd(III)-F distance  $0.72 \pm 0.02$  nm (the average of all three  $\text{CF}_3$  atoms) is in good agreement with the value from X-ray data. The downfield shift ( $-0.33$  ppm) of *N*-trifluoroacetyl- $\beta$ -glucosamine (compared with the upfield shift of 1 ppm for the  $\alpha$ -form) may be explained on the basis of electrostatic effects of Try 108 (and Tyr 63) on the  $\text{CF}_3$  group, since residue 108 is known to tilt (L.N. Johnson, private communication) when binding occurs.

It has been suggested that Mn(II) binds at the active site [9], presumably in a similar way to Gd(III). The different paramagnetic probes, Mn(II) and Gd(III) can thus provide a more rigorous test of the validity of this mapping procedure. As for Gd(III), the correlation time of the Mn(II)/lysozyme complex was estimated from measurements of the solvent-water relaxation rates. Using these values in the calculation of distances of selected nuclei in methyl-*N*-fluoroacetyl- $\beta$ -glucosamide from the Mn(II) or Gd(III) site we have

obtained the following results (average of five experiments):

Nucleus	Distance (in nm)	
	Mn(II)	Gd(III)
$-\text{CH}_2\text{F}$ (proton)	0.58	0.55
$-\text{OMe}$ (proton)	0.52	0.49
$-\text{CH}_2\text{F}$ (fluorine)	0.57	0.51

The distances are in good agreement but the consistently lower distances from Gd(III) may reflect a slight difference in the mode of binding between Gd(III) and Mn(II). More detailed studies are necessary to establish this point and these are currently in progress. The results thus confirm that, as with *N*-acetylglucosamine [2,10], the fluoroanalogues bind in the C sub-site of lysozyme.

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Table 1  
Proton relaxation data for methyl 2-deoxy-2-fluoroacetamido- $\beta$ -D-glucoside ( $\beta\text{MeGluNAcF}$ ) and lysozyme in the presence of Mn(II) and Gd(III) (pH 5 nominal,  $22^\circ$ ).

Metal ion		$\beta\text{MeGluNAcF}$ (mM)	Lysozyme (mM)	Reference Group	$\frac{1}{T_2\rho}$ (Hz)	Distance (nm)
Mn(II)		190	2.52	$-\text{CH}_2\text{F}$	0.88	0.57
	Gd(III)	190	2.52	$-\text{CH}_2\text{F}$	0.74	0.56
Mn(II)		179	2.41	$-\text{CH}_2\text{F}$	1.49	0.59
	Gd(III)	179	2.41	$-\text{CH}_2\text{F}$	1.0	0.57
Mn(II)		172	2.31	$-\text{CH}_2\text{F}$	2.15	0.58
	Gd(III)				1.78	0.55
Mn(II)		190	2.52	$-\text{OCH}_3$	1.89	0.50
	Gd(III)	190	2.52	$-\text{OCH}_3$	1.67	0.47
Mn(II)		179	2.41	$-\text{OCH}_3$	2.62	0.54
	Gd(III)	179	2.41	$-\text{OCH}_3$	2.22	0.50
Mn(II)		172	2.31	$-\text{OCH}_3$	4	0.52
	Gd(III)	172	2.31	$-\text{OCH}_3$	2.94	0.50

The NMR methods used are described in [5].

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