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N-Fluoroacetyl-D-glucosamine as a Molecular Probe of Lysozyme Structure Using [^{19}F]Fluorine-Nuclear-Magnetic Resonance Techniques

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1. The synthesis and nuclear-magnetic-resonance spectra of *N*-fluoroacetyl-D-glucosamine are described, together with ^{19}F chemical shifts for some monosaccharides substituted with fluorine at different positions.

2. The sugar derivative *N*-fluoroacetyl-D-glucosamine is inhibitory towards lysozyme, 50% of full inhibition being given by about 15 mM.

3. At pH 5.4, addition of this sugar derivative to lysozyme solutions results in a concentration-dependent broadening and down-field shift in the ^{19}F-resonance, of both anomeric forms. The α-anomeric form is shifted more than the β-form.

Application of nuclear-magnetic resonance offers a number of approaches for the study of enzymes, including the observation of protein resonances by high-resolution spectra, of substrate resonances in the investigation of conformations of the substrates in solution; and indirect methods such as studies of paramagnetic probes in (proton) relaxation enhancement of the solvent water protons.

The advantages of studies of substrates lie in the availability of material and in the relative ease with which most commercial nuclear-magnetic resonance spectrometers can detect 10 mM proton concentration. When a substrate is bound to an enzyme, a change in the chemical shift of substrate protons may occur. If there is rapid chemical exchange between the bound and free environment the observed shift will be the weighted average of the two environments. Unfortunately proton chemical shifts in different molecular environments are small in normal diamagnetic molecules. The use of paramagnetic probes to amplify this shift by means of either contact or pseudo-contact interaction with substrate protons is often necessary to produce more measurable effects.

The binding of methyl *N*-acetyl-α and β-glucosamine (α and β MeGlcNAc) to lysozyme has been observed [1–3] to produce a shift of the acetamido-CH_3 protons of +0.5 ppm for the fully-formed β-MeGlcNAc · lysozyme complex. Addition of Eu(III) to this system results in shifts arising from dipolar interactions between the substrate nuclei and unpaired electrons of Eu(III) such that the measured values become of the order of 2 ppm [3a]. This shift is termed a pseudo-contact shift and, for a given geometry, the magnitude of this pseudo-contact shift is independent of the substrate nucleus, (neglecting any through-space interactions of the metal ion with the electron clouds around the nucleus), provided that the nucleus does not cause changes at the metal-binding site of the enzyme. By contrast, chemical shifts differ considerably from one nucleus to another. Thus, for example, ^{19}F chemical shifts are generally an order of magnitude greater then those of protons.

In this paper, we have attempted to explore the possibilities of using ^{19}F as a covalent probe by observation of the changes in ^{19}F chemical shifts when *N*-fluoroacetyl-D-glucosamine (GlcNAcF) is bound to lysozyme.

This fluorine-containing sugar is one of the large number of fluorocarbohydrates to have been synthesized chemically, (reviewed by Kent [4]) information about whose biological activities is only just becoming available [5]. Some of these results have already been published in a preliminary form [6, 7].

MATERIALS AND METHODS

1,3,4,6-Tetra-O-*acetyl*-N-*fluoroacetyl*-*β*-D-*glucosamine (I)*

Tetra-*O*-acetyl-β-D-glucosamine [8], (m.p. 186 °C; $[\alpha]_D^{24}$ + 1.3 °C; *c*, 1, $CHCl_3$; 3.8 g) was dissolved in dry chloroform (47.5 ml) and dry pyridine (7.5 ml) at 2 °C. Dicyclohexylcarbodiimide (1.9 g) was added, followed by fluoroacetic acid (1.6 ml). After 16 h at 2 °C, the filtered solution was washed with water, dilute hydrochloric acid and again with water. After

Unusual Abbreviations. GlcNAcF, *N*-fluoroacetyl-D-glucosamine; αMeGlcNAc, methyl 2-acetamido-2-deoxy-α-D-glucoside; βMeGlcNAc, methyl 2-acetamido-2-deoxy-β-D-glucoside.

Enzyme. Lysozyme or mucopeptide *N*-acetylmuramyl hydrolase (EC 3.2.1.17).

being dried ($MgSO_4$), the solution was concentrated to dryness and the resulting *N*-fluoroacetate was recrystallised (EtOH), yield 2.6 g, 65%; m.p. 185 °C, $[\alpha]_D^{20} = 1.2$ °C (*c* 1, $CHCl_3$), R_F 0.77 (thin-layer chromatography). Analysis: found, C, 47.2; H, 5.4; F, 4.3; N, 3.6; calc. for $C_{16}H_{22}FNO_{10}$: C, 47.2; H, 5.4; F, 4.7; N, 3.4%. Previous [11] m.p. was 176–9 °C.

N-*Fluoroacetyl-D-glucosamine (GlcNAcF, II)*

Deacetylation of I. The tetra-*O*-acetate (I, 0.1 g) in dry methanol (1.5 ml) at 0 °C was treated with magnesium methoxide (1.1 ml, 5% w/v in methanol). After 1 h about 10 vol of Zeo-Karb 225 resin (H^+ form, methanol washed) was added and stirred for 2 h. The filtered solution was evaporated, some unchanged glucosamine removed and the product was separated chromatographically on a cellulose column (29×3.5 cm) using the water-poor phase of butan-1-ol–ethanol–water (4:1:5, v/v/v). The product GlcNAcF obtained after 230 ml has passed through the column was recrystallised from dry methanol. Yield 20 mg, m.p. 161–163 °C $[\alpha]_D^{25} + 22.8$ °C (after 3 h *c* 0.3, H_2O), R_F 0.45 (thin-layer chromatography), 0.24 (paper) (*cf.* [9] m.p. 189–192 °C; $[\alpha]_D^{20} + 31$ °C (2 h *c* 1, H_2O), relative retention time t_R 1.2 (relative to mannitol).

Direct Synthesis (contributed by Mr P. F. Daniel). D-Glucosamine hydrochloride (12.5 g) and sodium fluoroacetate (6.3 g) in water (60 ml) was mixed slowly with a solution of dicyclohexylcarbodiimide (20 g) in pyridine (165 ml). After 24 h at room temperature, the solution was diluted with water (600 ml), filtered, extracted with ether (4×100) and the aqueous solution was concentrated to dryness. The residue was extracted with hot dry ethanol (7×100 ml), and filtered through Celite. To this 400 ml acetone was added and the unchanged glucosamine was removed. Concentration of the filtrate gave the product (GlcNAcF, II) which after recrystallisation from dry methanol had m.p. 161–2 °C (alone or mixed with the product of the previous experiment), yield 19.9%, $[\alpha]_D^{24} +$ 23.4 °C (3 h, *c* 1, H_2O) R_F 0.45 (thin layer chromatography), 0.24 (on paper). Analysis: found, C, 40.0; H, 6.1; F, 7.7; N, 5.8; calc. for $C_8H_{14}FNO_6$: C, 40.2; H, 5.9; F, 7.9; N, 5.9% relative retention time to mannitol 1.21.

Fluoroacetamide (III)

This was prepared by ammonolysis of ethyl fluoroacetate and was recrystallised from ethanol; m.p. 106 °C.

Difluoracetamide

This was prepared as (III) from ethyl difluoroacetate and recrystallisation from methanol, m.p. 50–51 °C [10].

Trifluoroacetamide

This was obtained from Koch-Light Laboratories Ltd (Colnbrook, Bucks.), recrystallised from dry ethanol and sublimed *in vacuo*, m.p. 74.6 °C [11].

Hen-Egg Lysozyme

Crystalline enzyme (LY 9FA, Worthington Biochemical Corp.) was used.

Enzyme Kinetics

The action of potential inhibitors on lysozyme was investigated by a modification of the technique of Neuberger and Wilson [12] using a substrate, suspensions of *Micrococcus lysodeikticus* (Lot no. 24B 1460, Sigma Chem. Co.) in 0.1 M sodium-citrate buffer pH 5.5. The probe molecule was pre-incubated with enzyme for 2 min at 30 °C before addition into substrate. Polypropylene tubes were used throughout for incubations and quartz cells for spectrometry.

Paper Chromatography

This was performed by downward elution on Whatman No. 1 paper using the water-poor phase of butan-1-ol–ethanol–water (4:1:5, v/v/v). Reducing sugars were detected using silver nitrate in acetone and ethanolic sodium hydroxide [13].

Thin-Layer Chromatography

This was performed on cellulose-layered (0.025 mm) plates (5×20 cm) eluting with the same solvent as in the foregoing procedure.

Gas-Liquid Chromatography

N-Acylglucosamines were analysed, as trimethylsilyl derivatives on a column (200 cm) of Diataport-S-100 1%-SE 30 as described previously [16] using mannitol as standard.

Optical Rotations

These were measured on a Bendix-Ericsson photoelectric polarimeter (Type 143A) having a full-scale deflection of 0.0115 ° in sodium light (calibrated with sucrose).

^{19}F-*Nuclear-Magnetic-Resonance Spectra*

These were recorded on a Bruker MFX-90 operating at 84.6 MHz and at 27 °C. The shifts were measured with respect to ^2H_2O used as an internal locking signal and trifluoroacetone was added as an internal standard. Solutions were made up in ^2H_2O and 0.1 M imidazole buffer ($p^2H = 5.4$).

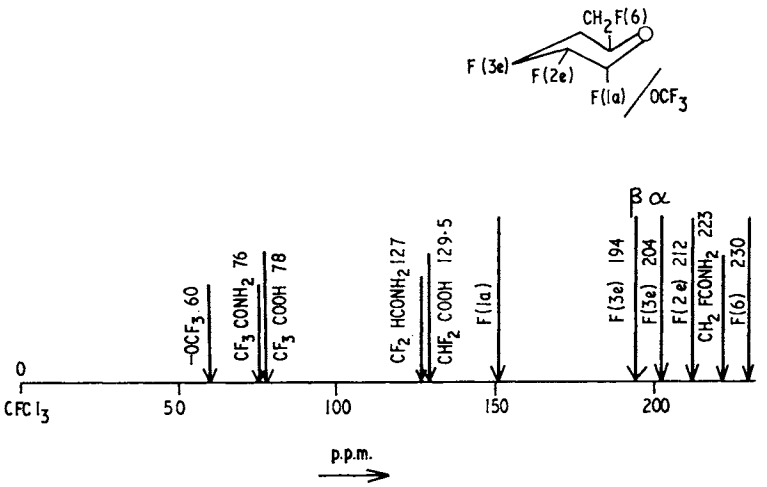

Fig. 1. *Summary of values of ^{19}F shifts (φ_c) in fluoromonosaccharides.* F(1a) *etc.* refer to the position of substitution of F in a variety of fluoromonosaccharides [16]

Toxicity

Until detailed information is obtained, GlcNAcF and similar compounds should be treated with considerable caution as potential toxic hazards. GlcNAcF itself is toxic in mice and it may be converted into fluoroacetic acid, the toxic properties of which are well known [14, 15]. It is emphasized that fluoracetamide, likewise is very poisonous though difluoroacetamide and trifluoroacetamide are less so.

RESULTS AND DISCUSSION

In an earlier exploration [16] we reported extensive ^{19}F chemical shifts (φ_c) in a variety of differently substituted fluoromonosaccharides. As expected with fluorine nuclei, the chemical shifts vary considerably depending on their environment in the monosaccharide. For instance an axial fluorine nucleus at C-1 has a chemical shift (relative to $CFCl_3$) of approximately 150 ppm, while an equatorial fluorine at C-2 (F2e) has $\varphi_c \approx 212$ ppm. With an equatorial fluorine attached to C-3, it has been possible to observe different shifts for the α and β form of the monosaccharide and the β form has the lower shift ($\varphi_c = 194$ ppm). The overall pattern of chemical shifts is summarised in Fig. 1 together with the shifts of several derivatives of trifluoroacetic acid which can be used as suitable standard reference compounds. The chemical shifts are however, considerably larger than those for the corresponding protons, which only extend over a range of ≈ 5 ppm. This fact, coupled with the size of the fluorine nucleus (almost identical to that of an —OH) make it an attractive possibility in the design of suitable probes for enzymes.

N-Fluoroacetylglucosamine (GlcNAcF) has resonances centered about $\varphi_c = 228$ ppm, consistent with the location of F outside the pyranose ring. In 100% ^2H_2O the spectrum consists of two sets of triplets ($J = 46$ Hz) separated by 0.46 ppm corresponding to the α and β anomers. The assignments shown in Fig. 2 are based on a comparison with the ^{19}F-nuclear-magnetic-resonance spectra of α- and β-methyl *N*-fluoroacetylglucosamines [17]. It will be noted that the ratio of α to β forms is approximately 1:1. In 25% ^2H_2O, the spectrum of each anomer appears as two triplets separated by 0.13 ppm in the ratio 1:3, corresponding to the $-N^2H$ or $-N^1H$ form of GlcNAcF. At 80 °C the two sets of resonances coalesce. The neglect of this complication has led us to a revised interpretation of the spectrum of GlcNAcF [6]. The complete spectrum (at 26 °C) in 25% ^2H_2O is shown in Fig. 3.

Binding of GlcNAcF to Lysozyme

The kinetic measurements show that GlcNAcF is inhibitory towards lysozyme; 50% of full inhibition being given by about 15 mM. This value is similar to that for *N*-acetyl-α-glucosamine.

The addition of GlcNAcF to lysozyme also results in changes in its ^{19}F spectrum. All the resonances shift downfield. However, the resonances of the α anomer shift noticeably more than those of the β anomer. Fig. 2 illustrates the ^{19}F spectrum for a solution at at p^2H 5.4, containing 3 mM lysozyme and 200 mM GlcNAcF.

Raftery and colleagues found from proton-magnetic-resonance-shift measurements on α and β GlcNAc and α and β MeGlcNAc in the presence of lysozyme, that the dissociation constants for the fully formed complexes are 30 mM. In this case, the methyl-proton resonances can be observed and, as there is no hyperfine coupling to the methyl group, the methyl

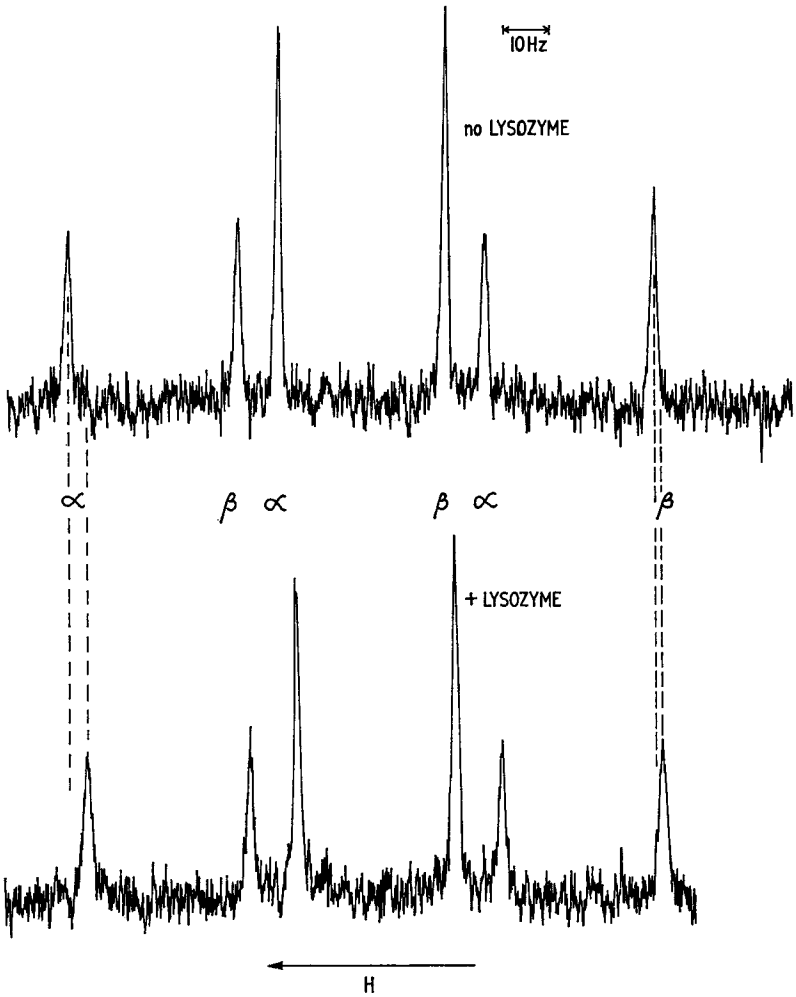

Fig. 2. *^{19}F shifts for α and β GlcNAcF in the presence and absence of lysozyme.* Conditions: lysozyme (3 mM), GlcNAcF (200 mM), imidazole buffer, 99.8% ^2H_2O, pH (nominal) 5.0, 26 °C

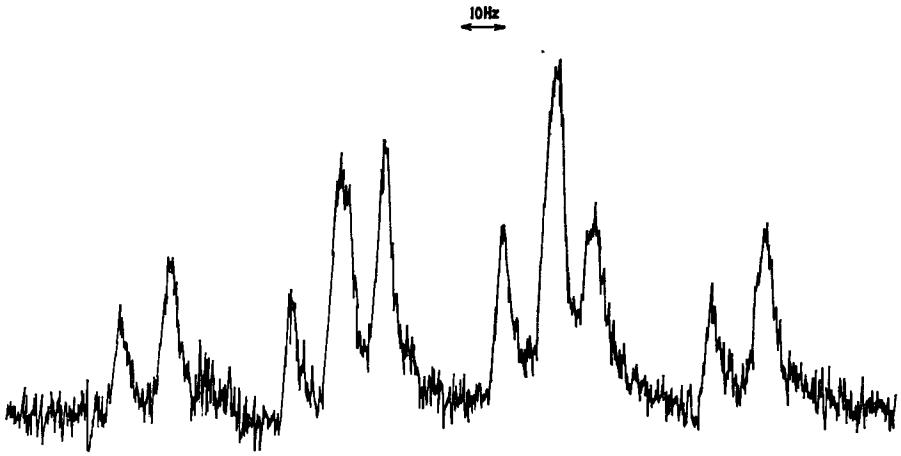

Fig. 3. *^{19}F spectrum of α and β GlcNAcF in 25% ^2H_2O.* Conditions: GlcNAcF (200 mM), imidazole buffer, 25% ^2H_2O, pH (nominal) 5.0, 26 °C

resonances afford a highly favourable probe with which to monitor the binding. If the binding constants to lysozyme are of the same order of magnitude with GlcNAcF, then accurate binding data can really only be obtained by studying concentrations of GlcNAcF in this range. Unfortunately the large number of hyperfine lines observed in the nuclear-magnetic-resonance spectrum means that, with the instrumentation available to us at present, concentrations 200 mM GlcNAcF have to be used for good signal-to-noise ratios but future developments should enable this figure to be decreased dramatically. Of course the concentration of lysozyme can be increased such that measurable effects can be obtained but then problems of enzyme aggregation have to be considered.

If, however, we assume here that the dissociation constants of the GlcNAcF · lysozyme complexes are 30 mM, then we can arrange experimental conditions such that all the enzyme exists as the complex. In such a case the ^{19}F chemical shift of the complex (Δ) relative to the free GlcNAcF can be obtained from the relationship:

$$\delta = P_M \Delta$$

where δ is the observed ^{19}F chemical shift and P_M is the fraction of total GlcNAcF that exists as the complex. The above equation assumes that there is rapid chemical exchange of the free and bound forms of GlcNAcF, such that an average chemical shift of the two environments is obtained. The values of Δ obtained here are 1.9 ppm and 3.8 ppm for the β- and α-GlcNAcF · lysozyme complexes, respectively, indicating that the environment in the enzyme of these two anomers is different.

A similar conclusion has been obtained from a study of the chemical shifts of the acetamido-methyl protons in the α- and β-*N*-acetyl-glucosamine · lysozyme complexes in which the shifts are $\Delta = 0.68$ ppm and $\Delta = 0.51$ ppm respectively [2]. We note however that these shifts are upfield.

The values of Δ for the fluorinated compounds though larger than those of the protonated analogues are not as large as might have been expected in view of the large chemical shift generally observed with fluorine compounds. For example, the extreme sensitivity of fluorine chemical shifts to environments is well illustrated by the difference in ^{19}F shift (0.13 ppm) for the N^1H fluoroacetyl glucosamine compared with that for the N^2H compound and again for the difference in chemical shift (≈ 5 ppm) of the α and β anomers of 3-fluoro-monosaccharides (see Fig. 1). However, both of these are examples of chemical shifts which must have their origin in effects transmitted through chemical bonds. The relatively small chemical shifts obtained here for the GlcNAcF · lysozyme complexes may be a consequence of through-space interactions (*e.g.* ring-current effects) causing these shifts.

It is well-known that *N*-acetylglucosamine (GlcNAc) is a competitive inhibitor of lysozyme, though not a powerful one, and it is of interest that inhibition seldom exceeds 50% of full activity, irrespective of GlcNAc concentration. Potentially, the inhibitor may occupy any of the sub-sites of the enzyme though binding studies [17] by X-ray diffraction and then using proton-magnetic-resonance techniques [1,2,18] suggest that the sugar associates preferentially with sub-site C. This appears to be also the case with the more powerful oligosaccharide inhibitors which have *N*-acetylglucosamine as the terminal reducing sugar [18]. The much greater sensitivity of ^{19}F-magnetic-resonance parameters, in contrast to protons, indicates that F-substituted glucosamine derivatives (including oligosaccharides) may prove to be much more effective probes of specific binding sites than unsubstituted sugars.

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