A ^{19}F Nuclear Magnetic Resonance Study of the Binding of Trifluoroacetylglucosamine Oligomers to Lysozyme†

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ABSTRACT: The binding of *N*-trifluoroacetyl-D-glucosamine and its chitobiose, chitotriose, and chitotetraose analogs to hen egg-white lysozyme was studied using ^{19}F nuclear magnetic resonance. In addition, the dissociation constant K_S of the chitotriose analog–lysozyme complex was measured as a function of pH using ultraviolet difference spectral techniques. The *N*-trifluoroacetyl group ^{19}F nuclear magnetic resonances of these sugars underwent a change in chemical shift upon binding to lysozyme. It was possible to observe directly the ^{19}F nuclear magnetic resonance spectrum of the chitotriose analog–lysozyme complex. A pH study of the chitotriose analog–lysozyme ^{19}F nuclear magnetic resonance spectrum revealed that the chemical shift of the nonreducing end trifluoroacetyl group changed due to the ionization of Asp-101. The chemical shift of the reducing end trifluoroacetyl group also changed due to the ionization of Asp-101, and, in addition, changed due to the ionization of Glu-35. The change in chemical shift of the reducing end trifluoroacetyl group upon ionization of Asp-101 is interpreted as being caused by a translation of the bound chitotriose analog inhibitor relative to the binding cleft of the enzyme upon formation of a hydrogen bond to the ionized Asp-101.

It has previously been shown that the proton magnetic resonance spectrum of the acetamido methyl protons of various *N*-acetylglucopyranosides is affected by the presence of lysozyme (Dahlquist and Raftery, 1968, 1969). Both chemical shifts and line broadenings have been observed, depending on the compound studied and the conditions used. In favorable cases, it has been possible to measure the chemical shift of inhibitor protons in their binding site(s) on the enzyme. The difference, Δ, between the chemical shift of bound inhibitor proton(s) and that of the inhibitor proton(s) free in solution is a measure of the electrical and magnetic environment of the enzyme binding site.

If a nucleus can exist in either of two chemical environments, A and B, with resonant frequencies ω_A and ω_B, an exchange of the form

$$A \underset{K_{BA}}{\overset{K_{AB}}{\rightleftharpoons}} B$$

can profoundly affect the nuclear magnetic resonance (nmr) spectrum of this nucleus (Pople *et al.*, 1959). Two limiting cases can be easily analyzed. In the so-called slow-exchange limit, where K_{AB} and K_{BA} are *both* small compared to the separation in frequencies, K_{BA}, $K_{AB} \ll (\omega_A - \omega_B)$, two separate lines are observed, centered at ω_A and ω_B, and with line width given by $T_{2A}^{-1} = T_{2A_0}^{-1} + K_{AB}$, and $T_{2B}^{-1} = T_{2B_0}^{-1} + K_{BA}$, respectively, where $T_{2_0}^{-1}$ is the width in the absence of exchange. In the fast-exchange limit, where K_{AB} and K_{BA} are *both* large compared to the frequency difference, K_{AB}, $K_{BA} \gg (\omega_A - \omega_B)$, a single line is observed at a frequency of $\omega = P_A\omega_A + P_B\omega_B$ and with line width

$$T_2^{-1} = P_A T_{2A}^{-1} + P_B T_{2B}^{-1} + P_A^2 P_B^2(\omega_A - \omega_B)^2(K_{AB}^{-1} + K_{BA}^{-1})$$

P_A is the fraction of nuclei in state A. At intermediate exchange rates, the equation describing the nmr spectrum is much more complicated (Pople *et al.*, 1959).

In the case of enzyme–inhibitor binding, the exchange between free and bound forms of the inhibitor is described by the equilibrium

$$E + S \underset{K_{diss}}{\overset{K_{form}}{\rightleftharpoons}} ES$$

$$K_S = \frac{K_{diss}}{K_{form}} = \frac{[E][S]}{[ES]}$$

In many instances, K_{form} and K_{diss} are large enough compared to the difference in frequencies between the two inhibitor forms for the fast-exchange limit to be valid. The inhibitor nucleus is then shifted from its position in the free form by an amount δ, given by the equation $S_0 = (E_0\Delta/\delta) - K_S - E_0$, where E_0 and S_0 are the total concentrations of enzyme and inhibitor (Raftery *et al.*, 1968).

We report here some preliminary results on the binding of trifluoroacetylglucosamine (Glc-NTFAc) oligomers to lysozyme.[1] We have studied the ^{19}F magnetic resonance spectrum of (Glc-NTFAc), $(Glc\text{-}NTFAc)_2$, and $(Glc\text{-}NTFAc)_3$ in the presence of lysozyme under a number of different conditions, and have also measured the dissociation constant K_S of the $(Glc\text{-}NTFAc)_3$–lysozyme complex as a function of pH, using ultraviolet (uv) difference techniques (Dahlquist *et al.*, 1966). It was possible to observe directly the ^{19}F nmr spectrum of the $(Glc\text{-}NTFAc)_3$–lysozyme complex, using conditions under which the inhibitor was nearly 100% in the bound form. Thus, Δ_1, Δ_2, and Δ_3, the chemical shift differences of the reducing end, middle, and nonreducing end trifluoroacetamido group fluorine nuclei, respectively, could be measured directly in the enzyme–inhibitor complex.

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[1] Trifluoro-*N*-acetylglucosamine and its oligomers will be abbreviated by $(Glc\text{-}NTFAc)_n$. *N*-Acetylglucosamine and its oligomers will be abbreviated by $(Glc\text{-}NAc)_n$.

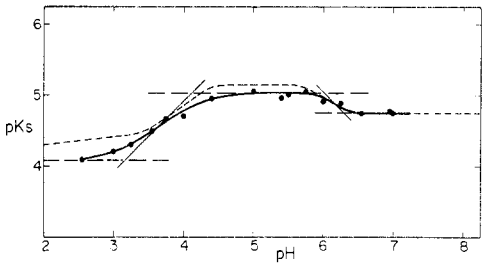

FIGURE 1: Solid line: plot of pK_s *vs.* pH for the $(Glc\text{-}NTFAc)_3$–lysozyme complex. Dashed line: similar plot for $(Glc\text{-}NAc)_3$ binding to lysozyme (Dahlquist *et al.*, 1966).

Results

Ultraviolet Binding Study of $(Glc\text{-}NTFAc)_3$. It is of interest to know whether substituting fluorines for hydrogens on the acetyl group has any important effect on the binding of acetylated glucosamine oligomers to lysozyme. With this in mind, the dissociation constant of the $(Glc\text{-}NTFAc)_3$–lysozyme complex was measured as a function of pH using uv difference techniques (Dahlquist *et al.*, 1966). The difference spectra occurred at 2900 Å, and were similar in appearance to those previously observed using acetylated glucosamine oligomers. The results, plotted as pK_s *vs.* pH, are given in Figure 1. The data were analyzed by the method of Dixon (Dixon and Webb, 1964), and it was found that the binding of $(Glc\text{-}NTFAc)_3$ perturbed two ionizable groups in the enzyme. A group of $pK = 5.8 \pm 0.2$ in the free enzyme was perturbed to 6.3 ± 0.2 in the ES complex, and another group of $pK_a = 4.2 \pm 0.2$ in the free enzyme was perturbed to 3.2 ± 0.2 It has previously been found that a group with a pK_a of 4.2 in free lysozyme is perturbed to 3.5 upon binding to $(Glc\text{-}NAc)_3$, while another group with a pK_a of 5.8 is perturbed to 6.2 upon binding to $(GlcNAc)_3$ (Dahlquist *et al.*, 1966). The group with $pK_a = 4.2$ has been identified as Asp-101, while the group with $pK_a = 5.8$ has been identified as Glu-35 (Dahlquist *et al.*, 1966; Dahlquist and Raftery, 1968; Parsons and Raftery, 1970). The binding data obtained for $(Glc\text{-}NTFAc)_3$ agree almost exactly with that for $(Glc\text{-}NAc)_3$. At all pH values, K_S has very nearly the same value for the two compounds and the measured pK_a values are also nearly the same, except that the pK_a of Asp-101 is perturbed to a slightly lower value for $(Glc\text{-}NTFAc)_3$ than for $(Glc\text{-}NAc)_3$, possibly because of a slightly stronger hydrogen bond to Asp-101 (Blake *et al.*, 1967). These data indicate that, at least for the trisaccharide, substitution of fluorines for hydrogen does not appreciably affect the binding.

^{19}F *Nmr Spectra of Glc-NTFAc Oligomers.* The ^{19}F nmr spectra of Glc-NTFAc, $(Glc\text{-}NTFAc)_2$, and $(Glc\text{-}NTFAc)_3$ are shown in Figure 2. It is seen that the fluorine nucleus is much more sensitive to conformational changes than the proton is, since the reducing end trifluoroacetamido group ^{19}F's have measurably different chemical shifts in the α and β anomeric forms. The monomer peaks were assigned by observing the spectrum of freshly dissolved α-Glc-NTFAc (Ashton *et al.*, 1971). Only the peak to lowest field was present initially, while the peak to high field appeared after about 5 min. The unsplit resonance of the trimer was assigned to the nonreducing end acetamido group, while the doublet to somewhat higher field was assigned to the middle acetamido group resonances of the α and β anomeric forms in reference to the dimer spectrum.

Binding of Glc-NTFAc and $(Glc\text{-}NTFAc)_2$ *to Lysozyme.*

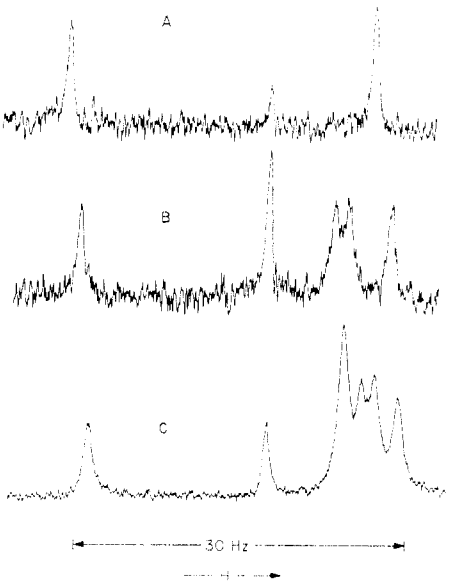

FIGURE 2: ^{19}F magnetic resonance spectra of: (A) Glc-NTFAc, (B) $(Glc\text{-}NTFAc)_2$, and (C) $(Glc\text{-}NTFAc)_3$. Spectra were recorded at 94.1 MHz in an aqueous solution of pH 5.0 and a temperature of 31°. The second peak from the left is due to trifluoroacetate anion used as an internal reference.

The analysis of the nmr shift data for Glc-NTFAc and $(Glc\text{-}NTFAc)_2$ in the presence of lysozyme was complicated by the presence of their anomeric forms. Therefore only a qualitative discussion of the results will be given.

The ^{19}F nmr resonance of α-Glc-NTFAc showed a large chemical shift to higher field in the presence of lysozyme at pH 5.5 and room temperature, while the peak due to β-Glc-NTFAc showed no chemical shift at any of the concentrations used. Either the β anomer does not bind nearly as strongly as the α, or it has a small chemical shift, Δ, when bound to the enzyme. Ashton *et al.* (1971) have recently studied the binding of α- and β-Glc-NTFAc to lysozyme in somewhat greater detail than is given here, and find an upfield shift of $\Delta = 78$ Hz and a dissociation constant of 9.1×10^{-3} mole/l. for α-Glc-NTFAc.

It was found that for $(Glc\text{-}NTFAc)_2$ the exchange $S + E \rightleftharpoons ES$ was not in the fast-exchange limit at room temperature and pH 5.5 with the spectral lines being broad. However, at 50° the lines sharpened up considerably and the nmr fast-exchange condition was satisfied. The ^{19}F resonance of the reducing end trifluoroacetamido group of α-$(Glc\text{-}NTFAc)_2$ showed a large upfield shift, while that of the β anomeric form again showed no measurable chemical shift at any of the concentrations of inhibitor used. The resonance due to the nonreducing end trifluoroacetyl group, to lowest field in the free sugar, moved to still lower field in the presence of lysozyme. Assuming roughly equivalent binding of the α and β anomers, $\Delta\alpha_1 \approx 80$–$150$ Hz and $\Delta_2 \approx -20$–30 Hz.

^{19}F *Nmr Studies of* $(Glc\text{-}NTFAc)_3$ *Binding to Lysozyme.* At room temperature the nmr spectrum of $(Glc\text{-}NTFAc)_3$ was relatively unaffected by the presence of a small amount of lysozyme, because the exchange $S + E \rightleftharpoons ES$ was in the slow-exchange limit. At a temperature of 65°, however, the lines were quite sharp, and presumably the fast-exchange limit was valid. It was necessary at 65° to carry out the binding study at pH 4 and to take the spectra very quickly to avoid complications due to enzymatic hydrolysis. The rate of hydrolysis under these conditions could be determined by observing the

TABLE I: Chemical Shift Data for the Trifluoroacetyl Group ^{19}F Resonances of the $(Glc\text{-}NTFAc)_3$–Lysozyme Complex, and the *N*-Acetyl ^1H Resonances of the $(Glc\text{-}NAc)_3$–Lysozyme Complex (in Hertz at 94.1 MHz and 100 MHz, respectively).

Substrate	*T*, °C	pH	$\Delta_{1\alpha}$	$\Delta_{1\beta}$	Δ_2	Δ_3
$(Glc\text{-}NTFAc)_3$	65	4.0	-57 ± 5	-29 ± 5	-38 ± 5	$-122 + 5$
$(Glc\text{-}NTFAc)_3$	10	9.0	−64	−53	−40	−130
$(Glc\text{-}NAc)_3$	65	9.7	+77		0	−9

nmr spectrum at various times after the temperature was raised, and in this manner it was found that a negligible amount of hydrolysis had occurred in the time it took to record the first nmr spectrum. The recent nmr study of the thermal denaturation of lysozyme by McDonald *et al.* (1971) shows clearly that an insignificant amount of lysozyme is denatured at 65° and pH 4, although denaturation is essentially complete at 75°. Since there was no splitting of the resonances due to the trifluoroacetyl groups of the nonreducing or middle peaks in the presence of lysozyme, the dissociation constants of the two anomeric forms must have been nearly the same. The results are shown in Table I.

Since the dissociation constant K_S is so small for the $(Glc\text{-}NTFAc)_3$–lysozyme complex, the inhibitor should be almost 100% bound in an equimolar solution of lysozyme and $(Glc\text{-}NTFAc)_3$ (3×10^{-3} M). The ^{19}F nmr spectrum of $(Glc\text{-}NTFAc)_3$ in such a solution will then be that of the ES complex, regardless of whether the exchange is fast or slow. In fact, it can be stated that as far as nmr is concerned, there is no exchange. The ^{19}F nmr spectrum of a solution of 3×10^{-3} M lysozyme and 3×10^{-3} M $(Glc\text{-}NTFAc)_3$ at pH 4.5 and a temperature of 10° is shown in Figure 3. At pH 4 the chemical shifts of the three lines agree well with those obtained from the high-temperature equilibrium study, and the assignments are as follows. The line to lowest field arises from the nonreducing end trifluoroacetyl group (Δ_3), the middle line arises from the reducing end trifluoroacetyl group (Δ_1), and the line to highest field arises from the middle trifluoroacetyl group (Δ_2). The ^{19}F spectrum was very similar from 0° up to 50°, illustrating that there was effectively no exchange, since there was very little free inhibitor present. The pH dependence of the three resonances was measured at a temperature of 10°, and is shown in Figure 4. It was found that the chemical shift of the middle trifluoroacetyl group is relatively pH independent, while both the reducing end and nonreducing end trifluoroacetyl groups showed a large break at a pH of 3.25. This is in agreement with the uv binding data, and the change in Δ_1 and Δ_3 at pH 3.25 is then due to the ionization of Asp-101. The large change in Δ_3 upon ionization of Asp-101 is not unexpected, since the nonreducing end trifluoroacetyl group is hydrogen bonded to Asp-101 (Blake *et al.*, 1967). However, the change in Δ_1 at the same pH value was not expected, since the reducing end of the molecule is so far removed from Asp-101. It is unlikely that any other group is causing this change, since there are only three titratable carboxyls in the binding site of $(Glc\text{-}NTFAc)_3$, Asp-101, Asp-52, and Glu-35, and only Asp-101 has a pK in the range 3.2 in the bound enzyme. Δ_3 shows no change in the pH region 4.5–9.0. The reducing end trifluoroacetyl group broadens at a pH of 6.0 and splits into a doublet by a pH of 7.0, indicating that the Δ's of the α and β reducing end groups are now nearly the same, or possibly are even more different than at lower pH. It was not possible to determine which peak was due to which anomer.

The reducing end peaks were also considerably broader at pH values of 7.0 and above, and thus less intense than the other two resonances. They were also much harder to saturate with high power levels, indicating that T_1 was much shorter, and that they were probably in a more tightly bound configuration than at lower pH values.

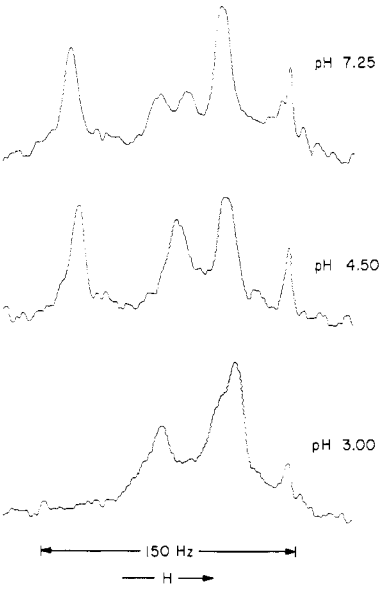

FIGURE 3: ^{19}F magnetic resonance spectra of solutions containing 3×10^{-3} M $(Glc\text{-}NTFAc)_3$ and 3×10^{-3} M lysozyme at 10° at various pH values. The resonance to highest field is due to the trifluoroacetate anion.

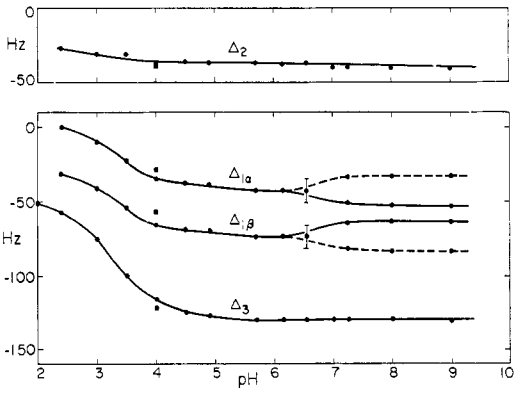

FIGURE 4: Chemical shift data for the trifluoroacetyl group ^{19}F resonance for the $(Glc\text{-}NTFAc)_3$–lysozyme complex (3×10^{-3} M) at various pH values. The temperature was 10°. The squares represent the chemical shifts of Table I measured at pH 4.0 and at a temperature of 65°.

Effect of Co^{2+} on the (Glc-NTFAc)$_3$–Lysozyme Complex. It is known that Co^{2+} (McDonald and Phillips, 1969) and the heavy metals Hg^{2+}, U^{4+} (Blake *et al.*, 1967), Eu^{3+}, Ho^{3+}, and Gd^{3+} (Morallee *et al.*, 1970) bind to the active site of the lysozyme molecule, most likely complexed with the catalytic carboxyl groups, Asp-52 and Glu-35. Therefore, the resonance due to the trifluoroacetyl group in site C should undergo a paramagnetic shift, while those due to trifluoroacetyl groups in sites A and B should be relatively unaffected.

The addition of Co^{2+} to a solution of 3×10^{-3} M lysozyme and 3×10^{-3} M (Glc-NTFAc)$_3$, at pH 5.5, and a temperature of 30° shifted the reducing end trifluoroacetyl group resonance upfield by about 20 Hz, leaving the other two resonances unaffected. The binding constant of Co^{2+} to lysozyme was measured to be about 1.5×10^{-2} M, in agreement with the data of McDonald and Phillipps (1969). These data verify that the reducing end of the sugar is indeed located in site C.

^{19}F Nmr Spectra of (Glc-NTFAc)$_4$–Lysozyme. The ^{19}F nmr of a solution containing 3×10^{-3} M lysozyme and 3×10^{-3} M (Glc-NTFAc)$_4$ was identical with the spectra of the (Glc-NTFAc)$_3$–lysozyme complex, with one additional peak at a position unshifted from the resonance due to the nonreducing end trifluoroacetyl group of free (Glc-NTFAc)$_4$. This indicates unambiguously that in the dominant binding mode (Glc-NTFAc)$_4$ occupies positions A, B, and C in the binding cleft, with the nonreducing end residue sticking out into solution.

Discussion

There are three different interactions which could lead to a chemical shift at the binding site, so that Δ is the sum of three terms (Buckingham *et al.*, 1960).

$$\Delta = \Delta_A + \Delta_W + \Delta_E$$

Δ_A is a shift due to the magnetic anisotropy of enzyme side chains near the inhibitor nucleus. In enzymes, this term will be caused primarily by the induced ring currents of aromatic side chains. Δ_W is due to van der Waals interactions between the inhibitor and the binding site of the enzyme, while Δ_E is caused by the presence of charged or highly polar groups in the binding site of the enzyme. Both of these interactions lead to a distortion of the electronic wave function in the vicinity of the nucleus studied, and thus modify the chemical shift. It should be noted that each of these terms is actually the difference between the interaction at the binding site and the corresponding interaction of the inhibitor molecule free in solution. Different nuclei at equivalent positions in closely analogous inhibitors will most likely experience different shifts $\Delta = \Delta_A + \Delta_W + \Delta_E$ upon binding in the active site of the enzyme. In particular, Δ_A should have the same value for ^{19}F in (Glc-NTFAc)$_3$ as it does for ^1H in (Glc-NAc)$_3$, if both inhibitors are bound identically to lysozyme. This is because Δ_A is due to local magnetic fields, which do not alter the electronic wave function of the inhibitor. Both Δ_W and Δ_E, on the other hand, would be expected to be an order of magnitude larger for ^{19}F than for ^1H, since the ^{19}F chemical shift is much more sensitive to changes in the electronic wave function than is the proton chemical shift (Pople *et al.*, 1959). A comparison of the shifts of ^{19}F and ^1H nuclei in (Glc-NTFAc)$_3$ and (Glc-NAc)$_3$ would then, in principle at least, allow one to separate the various effects causing the observed shifts.

Unfortunately, data at only a single pH value, 9.7, are available for the acetamido protons of (Glc-NAc)$_3$ (Dahlquist and Raftery, 1969). These shifts are compared with those of ^{19}F in (Glc-NTFAc)$_3$ at a pH of 9.0 in Table I. The large downfield shift of the nonreducing end acetamido ^{19}F resonance, compared to the much smaller one of the ^1H resonance, indicates that Δ_E is probably dominant. The change in Δ_3 of (Glc-NTFAc)$_3$ at pH 3.25 is probably caused directly by the negative charge formed upon ionization of Asp-101, as well as by the formation of a hydrogen bond between Asp-101 and the trifluoroacetyl group (Blake *et al.*, 1967). Both give rise to changes in the Δ_E term.

That the shift of the middle trifluoroacetyl group resonance is small and pH independent, while that of the middle acetamido group protons in (Glc-NAc)$_3$ is zero, is readily explained by the fact that this group is pointed away from the binding cleft of the enzyme, and is sticking out into the solvent (Blake *et al.*, 1967).

The behavior of the reducing end trifluoroacetyl group resonance is more complicated. The change in Δ_1 at a pH of 3.25 indicates that hydrogen bond formation to Asp-101 probably shifts the trimer relative to the binding cleft. Only a small translation of the inhibitor fluorines relative to the enzyme group causing the shift would be necessary to cause a change in Δ. It is unlikely that any other mechanism could affect the shift of this resonance upon ionization of Asp-101, since the reducing end trifluoroacetyl group is so far removed from Asp-101 in the binding cleft.

The change in the resonance at pH 6.0–7.0, probably due to Glu-35, is peculiar in that it is so small. A large change in the Δ of the acetamido group protons of β-methyl Glc-NAc was observed upon ionization of Glu-35. It is possible that Δ_A and Δ_E undergo rather large but opposite changes upon ionization of Glu-35. This cancellation of two terms would also explain the large difference between the shift of the reducing end acetamido group protons of (Glc-NAc)$_3$, and Δ_1 of (Glc-NTFAc)$_3$ at pH 9. Δ_A, probably due to the ring current of Trp-108, should contribute an equal amount (in parts per million) to the total shift of the two inhibitor resonances, and should furthermore be the dominant term for protons in (Glc-NAc)$_3$. An additional negative term Δ_W in the case of (Glc-NTFAc)$_3$ could then possibly cancel the Δ_A term and lead to a negative shift. It should again be emphasized that this sort of analysis is based on the assumption of identical binding, which at least seems likely in view of the close similarity of K_S and its pH dependence for the two inhibitors, as well as the finding that Co^{2+} bound in subsite D affects only the reducing end trifluoroacetyl group of (Glc-NTFAc)$_3$.

One additional point should be mentioned. The reducing end trifluoroacetyl group of both Glc-NTFAc and (Glc-NTFAc)$_2$ showed a large upfield shift, while that of (Glc-NTFAc)$_3$ was downfield. This indicates that the reducing end sugar of (Glc-NTFAc)$_3$ is really bound quite differently than that of Glc-NTFAc and (Glc-NTFAc)$_2$ in the binding cleft of lysozyme, although it is still in site C.

These experiments indicate the great sensitivity of the ^{19}F nucleus to the environment of binding sites on enzymes, and changes in this binding site caused by the ionization of groups therein.

Experimental Section

Uv Difference Spectra. The uv difference spectra of solutions containing 0.01 M phosphate buffer, 0.1 M NaCl, 2.48×10^{-5} M lysozyme, and concentrations of (Glc-NTFAc)$_3$ ranging from 10^{-3} M to 10^{-5} M were recorded at 15° on a Cary 14 uv spectrometer with a 0 to 0.1 absorbance slide-wire. The absorbance of the enzyme solutions *vs.* buffer was 0.94 *A*

at 280 nm. Pyrocell matched quarts uv cells of 10-mm path length were used, and the pH of the solutions was measured using a Radiometer Model 26 pH meter.

Nmr. All solutions used in the nmr measurements were 0.01 M in phosphate, 0.01 M in citrate, and 0.1 M in NaCl. Egg-white lysozyme (3 × crystallized, dialyzed, and lyophilized, Sigma Lot 6876) was used at a concentration of 3.3×10^{-3} M for the low-temperature $(Glc\text{-}NTFAc)_3$ study and for the Glc-NTFAc binding study, while a concentration of 3.3×10^{-4} was used in the high-temperature, fast-exchange study of $(Glc\text{-}NTFAc)_2$ and $(Glc\text{-}NTFAc)_3$. Spectra were recorded on a Varian XL-100-15 spectrometer operating at 94.1 MHz. For the 1:1 $(Glc\text{-}NTFAc)_3$ study, 12-mm, thin-walled tubes were used, while 5-mm tubes were used in the other studies. Both the free trifluoroacetic acid present and the H_2O heteronuclear lock were used as internal references, and the values obtained were always in agreement at a given temperature.

The trifluoro-*N*-acetylglucosamine oligomers were a gift of William Beranek, Jr. (W. Beranek and M. A. Raftery, to be published, 1971), and were prepared by trifluoro-*N*-acetylation of oligoglucosamines prepared by acid hydrolysis of N-deacetylated chitin.

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^{31}P and ^{13}C Nuclear Magnetic Resonance Studies of Nicotinamide–Adenine Dinucleotide and Related Compounds†

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ABSTRACT: The ^{31}P spectra of the reduced and oxidized forms of the following nucleotides have been obtained and analyzed: β-nicotinamide–adenine dinucleotide, nicotinamide–adenine dinucleotide phosphate, nicotinamide mononucleotide, and acetylpyridine–adenine dinucleotide. Also, spectra of α-nicotinamide–adenine dinucleotide, adenylic acid, and adenosine diphosphoribose were recorded. From the spectral differences between the reduced and oxidized nucleotides, as well as from the determination of the pK_a value of the phosphate group in the mononucleotides, it is postulated that in the oxidized nucleotides there is an electrostatic interaction between the positively charged nitrogen of the pyridine ring and a negatively charged oxygen of the diphosphate backbone. Using $BaCO_3$-^{13}C as a source of label, nicotinamide–adenine dinucleotide, enriched with ^{13}C in the carboxamide group, has been prepared *via* a series of chemical and enzymatic reactions. The ^{13}C nuclear magnetic resonance titration curve displays an apparent pK_a of 4, indicating that the chemical shift of the carboxamide carbon is influenced by protonation of the adenine ring of the dinucleotide.

Although proton magnetic resonance spectroscopy (pmr)[1] has now gained fairly wide use in the study of biologically important molecules, the use of other nuclei in such nmr studies has been rather limited. This has generally been because of the low sensitivity, in nmr experiments, of these other nuclei, their low natural abundance, or both. In certain cases the natural abundance problem can be overcome by enrich-

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[1] Abbreviations used are: pmr, proton magnetic resonance spectroscopy; nmr, nuclear magnetic resonance spectroscopy; NAD, β-nicotinamide–adenine dinucleotide; NADH, reduced β-nicotinamide–adenine dinucleotide; α-NAD, α-nicotinamide–adenine dinucleotide; NMN, nicotinamide mononucleotide; $NMNH_2$, reduced nicotinamide mononucleotide; APAD, acetylpyridine–adenine dinucleotide; APADH, reduced acetylpyridine–adenine dinucleotide; ADPR, adenosine-5′-diphosphoribose; AMP, adenylic acid; ATP, adenosine triphosphate; NADP, nicotinamide–adenine dinucleotide phosphate; NADPH, reduced nicotinamide–adenine dinucleotide phosphate.