AN NMR METHOD FOR CHARACTERIZING CONFORMATION CHANGES IN PROTEINS*

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Abstract

An NMR method for detecting and estimating the magnitude of conformation changes in proteins is described. By measuring both ^{19}F and ^{1}H chemical shifts in a $-CH_2F$ probe ring current shifts can be determined separately from shifts due to van der Waals interactions and electric fields. The method has been applied to lysozyme and a conformation change in a complex of the enzyme with β-methyl monofluoro-N-acetylglucosamine has been detected. The effect is due to the ionization of Asp 52, one of the catalytic groups, and the magnitude of the change is estimated to be about 1 Å. A mechanism for this conformation change is proposed.

NMR methods are particularly well suited for the study of conformation changes in proteins because of their great sensitivity to very subtle changes in environment. The interpretation of NMR results, however, is complicated by the large number of different interactions which can cause a chemical shift to change.

Since the chemical shifts of different nuclei respond differently to the various interactions in protein environments which can cause chemical shift changes, we considered it potentially informative to compare the chemical shifts of ^{19}F and ^{1}H on a CH_2F group in a protein environment. X-ray diffraction studies of lysozyme complexed with N-acetylglucosamine have shown that the N-acetyl methyl group is near the ring current of Try 108, and also near the two catalytic carboxyl groups Asp 52 and Glu 35 (1). We have studied the ^{19}F and ^{1}H NMR of the CH_2F group of β-methyl-2-fluoro-

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acetamido-2-deoxy-D-glucopyranoside (β-Me-Glc-NAcF) bound to lysozyme, and have detected conformation changes associated with the titration of the catalytic carboxyl groups Asp 52 and Glu 35.

CHEMICAL SHIFTS IN PROTEIN ENVIRONMENTS

The chemical shift in a protein environment can be separated into two contributions, $\sigma = \sigma$ (molecule) + Δ, where σ (molecule) is a chemical shift of the nucleus in the substrate molecule or amino acid residue in aqueous solution, and Δ is a shift due to the perturbing effects of the protein environment. Δ may have contributions from four sources (2, 3).

$$\Delta = \Delta_a + \Delta_w + \Delta_E + \Delta_c \qquad (1)$$

Δ_a arises from the ring current shifts of nearby aromatic residues in the protein, and may be either positive or negative depending on the orientation of the nucleus with respect to the plane of the ring. Their magnitudes for given orientations can be calculated with reasonable accuracy (4, 5). Such ring currents do not distort the electronic wavefunction on the nucleus under study and hence will cause the same chemical shift for any nucleus at the same position.

The last three terms in eq. (1) arise because of a distortion of the electronic wavefunction on the observed nucleus, and can be quite different for different nuclei in the same environment. Δ_w arises from van der Waals interactions with neighboring residues, Δ_E is due to a distortion caused by static electric fields, and Δ_c is due to specific bonding interactions with neighboring residues. Hydrogen bonding is the most common contribution to this last term in proteins.

The last three terms in eq. (1) are known to be an order of magnitude larger for ^{19}F on C-F bonds than for ^{1}H in C-H bonds (3). This is due to domination of ^{19}F chemical shifts by p-orbital electrons. ^{19}F chemical shifts cover a range of 400 ppm in organic compounds, compared

to about 10 ppm for proton chemical shifts. The solvent shifts of ^{19}F compounds are similarly an order of magnitude larger. The ^{19}F chemical shift in CHF_3 is fifteen times more sensitive to van der Waals perturbations, and at least four times more sensitive to electric field perturbations than the ^{1}H chemical shift in the same molecule (6). The difference in other molecules is even greater (3).

Thus, if both ^{19}F and ^{1}H chemical shifts on a $-CH_2F$ group are monitored, it would seem possible to distinguish between the various interactions causing a chemical shift change.

It is possible that in some cases the CH_2F group will be frozen as a certain rotomer, and thus the ^{19}F and ^{1}H nuclei will not experience identical environments, although the difference will be at most 2 Å.

METHODS

β-Me-Glc-NAcF was synthesized by methods similar to those used for Glc-NAcF (7). The proton NMR spectrum of β-Me-Glc-NAcF exhibits a doublet due to the CH_2F group 23 ppm downfield from H_2O with a J_{HF} value of 47 Hz. The ^{19}F NMR spectrum consists of a 1:2:1 triplet at 225 ppm to high field from trichlorofluoromethane. We measured the dissociation constant K_S and both ^{19}F and ^{1}H chemical shifts of β-Me-Glc-NAcF bound to lysozyme at various pH's by the fast exchange method (8) using a new referencing procedure (9).

All solutions contained 0.01 M phosphate, 0.01 M citrate, and 0.01 M NaCl. D_2O was used as solvent, the pD being the pH meter reading plus 0.4. A Varian XL-100-15 spectrometer was used for all NMR measurements.

RESULTS

The pH dependence of ^{19}F and ^{1}H chemical shifts for β-Me-Glc-NAcF bound to lysozyme is shown in figure 1, and the effect of pH on K_S is given

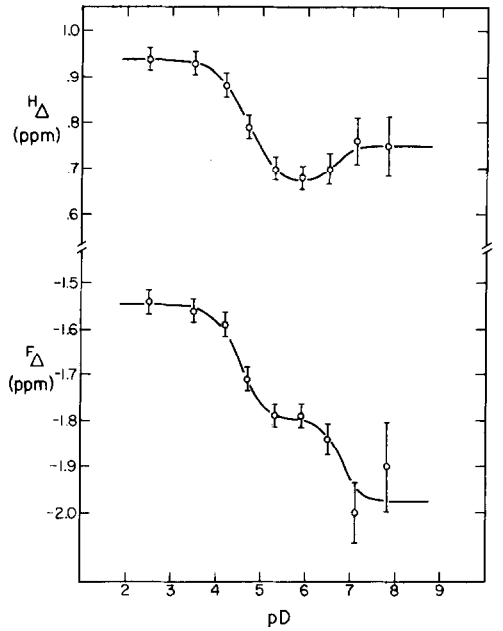

Figure 1. pD dependence of $^{H}\Delta$ and $^{F}\Delta$ for the CH_2F group in β-Me-Glc-NAcF bound to lysozyme at 35°C.

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in figure 2. The large break in K_S at pD = 6.5 is caused by the ionization of Glu 35 (10), while the break in ^{19}F and ^{1}H chemical shifts at pD = 4.5 is due to the ionization of Asp 52 (10).

The proton chemical shift $^{H}\Delta$, and the dissociation constant of β-

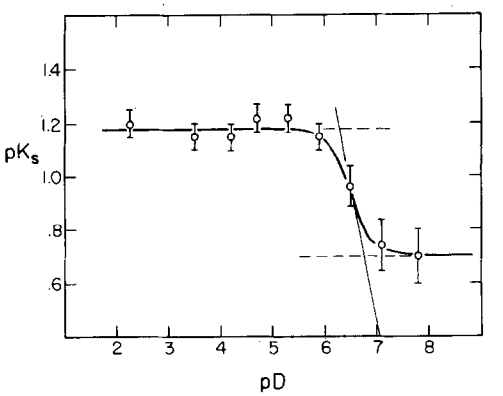

Figure 2. pK_S vs. pD plot for the β-Me-Glc-NAcF-lysozyme complex at 35°C.

Me-Glc-NAcF bound to lysozyme agrees very well with the same data for β-Me-Glc-NAc in the pH range 2-6 (11), when our referencing procedure (9) is used for both systems, indicating that the binding orientation of both inhibitors is very nearly the same. At higher pH's, however, the binding of β-Me-Glc-NAcF is somewhat different.

Sophianopoulos (12) and Studebaker, et al. (13) have suggested that dimerization of lysozyme at high pH's might affect the binding of Glc-NAc to site C. We have determined by U.V. difference measurements of K_s at low concentrations that there is insufficient dimerization up to pH 6 to affect our NMR results (9).

The T_2 values of the CH_2F protons of β-Me-Glc-NAcF in the presence of lysozyme are the same as those of the N-acetyl protons of β-Me-Glc-NAc in the presence of lysozyme (13). This indicates that the T_2 of the CH_2F protons in the β-Me-Glc-NAcF-lysozyme complex is quite long, ruling out the possibility that the CH_2F group is frozen in a specific rotomer. There is still a possibility, however, that although the rotation of the CH_2F group is rapid, one rotomer might be slightly favored.

DISCUSSION

Both X-ray (1) and NMR (8) evidence have shown that β-Glc-NAc binds to lysozyme very nearly the same as the reducing end pyranose ring of chitotriose, while α-Glc-NAc binds quite differently. Furthermore, β-Me-Glc-NAc has been shown to bind in a manner similar to β-Glc-NAc in site c (8), so we can interpret our results in terms of the X-ray crystal structure of the latter compound complexed with lysozyme (Fig. 3).

The overall magnitude of the two enzyme-bound shifts at pD = 5.5 are $^{H}\Delta = +0.7$ and $^{F}\Delta = -1.75$. Assuming free rotation of the CH_2F group, the ring current term for the two nuclei will be the same, $^{F}\Delta_a = {}^{H}\Delta_a$, so that the difference between the two must then be due to a contribution from the last three terms in eq. 1. Since $^{F}\Delta$ is not an order of magnitude

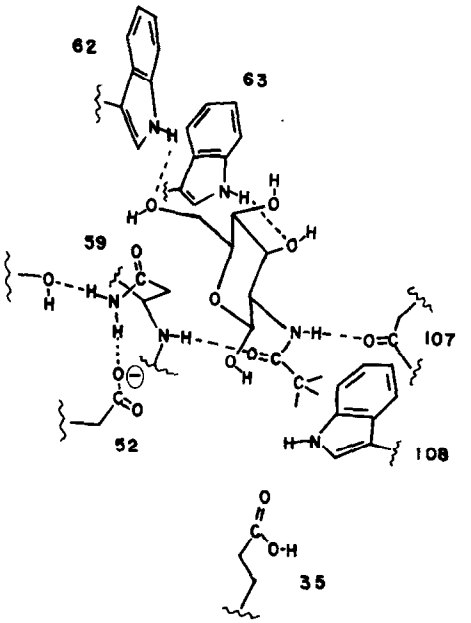

Figure 3. Binding of β-Glc-NAc to lysozyme. The hydrogen bonded network extending from Asp 52 to the inhibitor is shown as well as the position of the fluoroacetamido $-CH_2F$ group relative to tryptophan 108.

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larger than $^{H}\Delta$, the major contribution to $^{H}\Delta$ will be from $^{H}\Delta_a$. $^{H}\Delta$ at pH 5.5 agrees very well with a ring current shift of Try 108 calculated from the X-ray data (9). $^{F}\Delta$ at pH 5.5 must then be composed of an equal ring current term $^{F}\Delta_a = {}^{H}\Delta_a \approx 0.7$ ppm and a negative contribution from the last three terms in eq. (1) $^{F}\Delta_w + {}^{F}\Delta_E + {}^{F}\Delta_c = -2.60$.

The dependence of the chemical shifts on the ionization of Asp 52 is most interesting, since titration of this group does not cause a change in the K_s of either β-Me-Glc-NAc or β-Me-Glc-NAcF complexed with lysozyme. The chemical shifts of both nuclei changed the same amount in the ppm scale, indicating that the effect was caused by a conformation change of the CH_2F group relative to the ring current of Try 108. The NMR data rule out a direct charge effect on the chemical shifts as Asp 52 is ionized,

and in fact rule out a change in the contribution from the last three terms in eq. (1).

Since the X-ray crystal structure data (1) show a hydrogen-bond from the carboxyl group of ionized Asp 52 to the β-NH_2 group of Asn 59, which is in turn hydrogen-bonded through its α-amide NH to the carbonyl of the acetamido group of β-Me-Glc-NAc, we propose the following mechanism (figure 3): as Asp 52 is ionized it forms a hydrogen bond to Asn 59, pulling Asn 59, and causing it to pull the acetamido group toward a region of lower positive ring current field from Try 108. The magnitude of this translation can be calculated from Griessner-Prettre-Pullman tables (5) to be about 1 Å, (9) but the direction is not known uniquely. The absence of any change in K_S accompanying this conformation change puts a rather severe constraint on its nature. It is clear that the conformation change results from the hydrogen bonding network linking ionized Asp 52 with inhibitor.

The effect of Glu 35 is somewhat more difficult to evaluate quantitatively from the NMR data since the error in the measurements is large in the high pH range. The binding strength decreases considerably as Glu 35 is titrated, which indicates that the enzyme-substrate complex may undergo a conformation change, since the X-ray data indicates no direct bonding between Glu 35 and substrate in site C.

We have now observed conformation changes in the complexes of lysozyme with its substrates due to ionization of all three active site carboxyl groups, Asp 52, Glu 35, (9) and Asp 101 (15). It is possible that similar conformation changes occur in the actual catalytic process, and are important in lowering the energy of the transition state.

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