INTERACTION BETWEEN LYSOZYME AND ACETAMIDO SUGARS AS DETECTED BY PROTON MAGNETIC RESONANCE SPECTROSCOPY

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Recent X-ray studies on lysozyme and lysozyme-inhibitor complexes (Johnson and Phillips, 1965, Blake, 1966) have outlined the structure of the binding site in the crystal. Various techniques have been used to study the interactions involved in solution (Lehrer and Fasman, 1966, Glazer and Simmons, 1966); this paper indicates the use of N.M.R. in detecting binding of various acetamido sugars to lysozyme. In particular, it points to perturbation of the acetamido group as a result of binding, and possible interpretations of the results are discussed.

The 60MHz spectrum[2] of N-Acetyl D-glucosamine (NAG) is shown in Fig. 1A. The singlet at 141.6 cps is assigned to the methyl protons of the acetamido group (hereafter referred to as the acetyl signal). Doublets at 298 cps (partly obscured by the HDO peak) and 330 cps originate from the anomeric C-1 proton in its β- and α- orientation respectively. Thus a freshly prepared solution of β-NAG (synthesized according to Kuhn and Haber, 1953) showed only the 298 cps signal; within 70 min. it mutarotated to equilibrium showing

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[1] Facilities for this work were provided by Dr. O. Jardetzky. Address from 30th September 1966: Department of Surgical Research, Rikshospitalet, Oslo, Norway.

[2] All spectra were obtained in D_2O solution using a Varian DP60 instrument. Shifts were measured by the side band technique and are expressed in cps from hexamethyl disiloxane used as external reference.

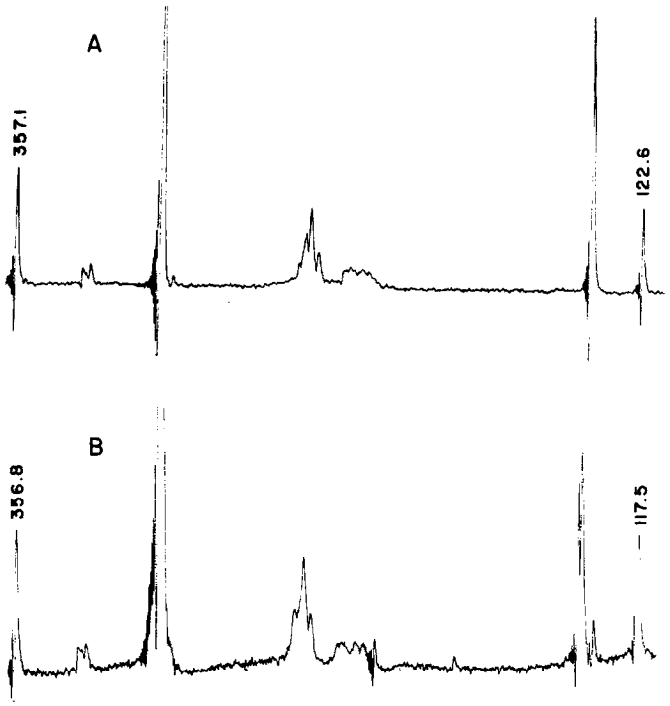

Fig. 1: N.M.R. spectrum of NAG alone (A) and in presence of 6% w/v lysozyme (B).

in addition the α- anomeric proton signal at 330 cps. The broad signals at 229 and 250 cps originate from the remaining ring protons.

In the presence of lysozyme (≥ 3% w/v) splitting of the acetyl signal occurs (Fig. 1B) giving two signals of approximately equal intensity and shifted slightly to higher field:[3]

Table I

Chemical shift of the acetyl signals in solutions of NAG (0.075M) containing lysozyme:

LYSOZYME CONCENTRATION (% w/v)	SHIFTS	
5 (heat-denatured)	141.6 (singlet)	
1	141.0 (")	
3	140.1	140.8
6	139.0	140.5
10	137.1	139.6

[3] Bulk susceptibility differences between solutions do not account for the observed shifts. Thus the methyl signal of acetone (used as internal reference) showed only a small (0.5 cps) shift to higher field on increasing lysozyme concentration from 2 to 10%.

Using 6% lysozyme and 0.075M β-NAG (cf. Table I), only a singlet at 140.1 cps is seen in freshly prepared solutions. However, as mutarotation proceeds (followed by the appearance of the anomeric proton signal at 330 cps) another acetyl signal appears at 138.7 cps, both attaining equal intensity in 60 min. Conversely if α-NAG was used, the acetyl singlet is initially at 138.7 cps with the weaker signal at 140.1 cps, and gradually increasing in intensity. The signals at 138.7 and 140.1 cps are thus assignable to the acetamido methyl protons of α- and β-NAG respectively.

A similar splitting of the acetyl singlet of N-Acetyl D-galactosamine was observed in the presence of lysozyme; Glazer and Simmons (1966) have shown that this compound binds to lysozyme on the basis of circular dichroism measurements. Lysozyme inactivated by heat or by treatment with N-Bromosuccinimide (Ramachandran and Rao, 1962) could not elicit the splitting effect, neither could a range of other enzymes and proteins. Chemical conversion of the NAG was also ruled out, indicating that a specific binding of NAG by the lysozyme was responsible. This is in accord with the dependence on lysozyme concentration (Table I). Again the observed shifts were pH dependent, being maximal between pH 5 and 6, minimal at pH 2 and 10 - further evidence for a binding process.

The results with NAG could be reproduced qualitatively using the α- and β-methyl glycosides of NAG[4] (these are stable compounds which do not interconvert in solution). Thus using 10% lysozyme and 0.05M α-methyl N-Acetylglucosaminide, the acetyl signal is observed at 138.0 cps. For the β- anomer, the corresponding signal is at

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[4] Prepared according to Zilliken et al., 1956.

137.1 cps. When both glycosides are mixed with lysozyme, two acetyl signals with a shift difference of approximately 1 cps are seen. The unperturbed signal for both anomers (in solutions containing 10% heat-denatured lysozyme) is at 141.0 cps. These observations show that the splitting of the acetyl signal seen in solutions of NAG containing lysozyme probably results from a different shift to higher field experienced by the acetamido methyl protons of each anomer on binding.

To account for the shift to higher field, it is postulated that the acetamido methyl group enters the diamagnetic region of an aromatic ring at the binding site(s). Assuming a rapid exchange rate between the bound and free forms of NAG, the observed shift will be a weighted average of the shifts for each species. In solutions containing 6% lysozyme and 0.075M NAG, approximately 4% of the NAG is bound, assuming only one binding site and a dissociation constant[5] of approximately 2×10^{-2}. For the observed shift of approximately 2 cps to higher field, the shift for the bound form is thus about 100 cps - a diamagnetic shift of about 40 cps from the unperturbed signal. This value is within that expected for shielding effects produced by aromatic rings (Johnson and Bovey, 1958). The presence of tryptophan residues at the inhibitor binding site is also well documented (Glazer and Simmons, 1966).

The different shifts seen for α- and β-anomers could then result from either differences in amounts bound, differences in orientation at the binding site, or even binding to different sites. This last possibility apparently exists for NAG binding in the crystal (Blake, 1966). It should be possible to distinguish some of these possibilities

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[5] S. Lehrer: personal communication.

by classical binding studies using the methyl glycosides of either NAG or N-Acetylgalactosamine. Further interpretations of the data do not seem warranted in the absence of information about binding constants and number of binding sites operative at these high lysozyme concentrations.

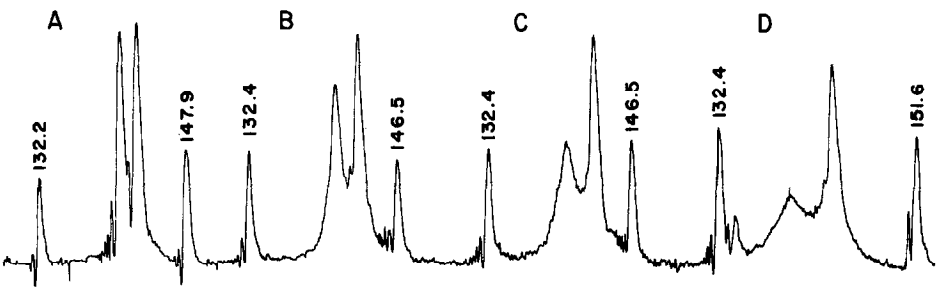

Fig. 2: Acetyl signal of N,N-Diacetyl chitibiose in presence of 0% (A), 0.5 (B), 2% (C) and 5% (D) lysozyme.

Interaction between lysozyme and N,N'-Diacetyl chitobiose could also be detected by N.M.R. Two acetyl signals (corresponding to the acetamido group on each ring) are observable, although no unequivocal assignment could be made. The effect of increasing lysozyme concentrations on the spectrum is shown in Fig. 2. It is not known whether the decrease in the intensity of the high field acetyl signal is accompanied by a change in its relaxation time, or whether splitting of the peak (by analogy with NAG) is occurring to give two broader signals. A study of this effect at 100 MHz seems warranted.

References

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