INTERACTION BETWEEN DIACETYLCHITOBIOSE METHYL GLYCOSIDE AND LYSOZYME AS STUDIED BY NMR SPECTROSCOPY

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Received October 23, 1967

In a previous report (Thomas, 1966) selective broadening of one of the two acetamidomethyl PMR signals of diacetyl chitobiose (diNAG) was seen in mixtures of the disaccharide with lysozyme. Interpretation of this observation was difficult for two reasons: firstly that mutarotation of the disaccharide (initially in the α-form) to give both the α- and β- anomers could complicate the binding, and secondly that an unambiguous assignment of the two acetamidomethyl signals could not be made. This report describes an experimental assignment of these signals, and clarifies the selective broadening effects seen in mixtures of lysozyme and either diNAG or its β-methyl glycoside.

At 60MHz in D_2O[2] the acetamidomethyl protons of diNAG

1) This work was supported by a NATO fellowship.
2) Spectra obtained using a Varian A60A instrument at Chemistry Department, Oslo University.

are seen as two sharp 3-proton signals at τ 7.92 and τ 7.95 (trimethylsilyl-propanesulfonate used as internal standard). A deuterated analog having a 2-N-trideuterioacetyl group (i.e. on the reducing ring) was prepared using methods first described for the corresponding monosaccharide (Leaback and Walker, 1957). Treatment of α-1-chloroheptaacetyl chitobiose (Osawa, 1966) with acidic nitromethane at 25°C gave the crystalline hydrochloride of 2-amino-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3,6-tri-O-acetyl-2-deoxy-α-D-glucopyranose. It had m.p. 216°C d., $[\alpha]_D^{20}$ +67° (c=0.7 H_2O) and analyzed correctly for $C_{26}H_{39}O_{16}N_2Cl$. Conversion of this salt to the free base, N-acetylation with p-nitrophenyl acetate-d_3 (prepared in turn from p-nitrophenol and acetic acid-d_3 using dicyclohexylcarbodiimide) in dimethylformamide, and de-O-acetylation with methanol-ammonia gave the required compound. It had an identical PMR spectrum to authentic diNAG, except that only one acetamidomethyl signal was observed at τ 7.92. The signal at τ 7.95 in diNAG must then originate from the 2-N-acetyl group attached to the reducing ring of the disaccharide.

The previous results (Thomas, 1966) with diNAG-lysozyme mixtures are thus partly clarified: the broadened signal is that from the acetyl group on the reducing ring of diNAG. However, the ambiguity with respect to mutarotation still remains. The α- and β-anomers could bind to different sites on the lysozyme molecule, as observed in the X-ray diffraction work on diNAG binding (Blake, C.C.F., et al., 1967). In the case of the N-acetylglucosamine anomeric mixture and lysozyme, this dual type of binding is shown as a splitting of the acetamidomethyl proton signal — probably due to the different shielding environments at each binding site. In the case of diNAG, splitting might be less than with NAG, or could be accompanied by broadening of the signals. Both effects would give a broad signal, and further studies at much higher resolution are required to distinguish them.

The situation might be simpler with binding of a glycoside of diNAG to lysozyme: such compounds do not mutarotate in aqueous solution, and binding to only one site becomes more probable. The β-methyl glycoside of diNAG was prepared from α-1-chloroheptaacetyl chitobiose and methanol using silver carbonate as

acid acceptor (Koenigs-Knorr reaction). De-O-acetylation of the product with sodium methoxide gave the required compound, m.p.303°C (needles, from MeOH) $[\alpha]_D^{20}$ -27° (c=1, H_2O). The PMR spectrum showed sharp singlets at τ 6.49 (methoxyl) and τ 7.92 and τ 7.97 (acetamidomethyl). The acetamidomethyl assignments are assumed to be identical with those for diNAG. The small shift in the high field signal (τ 7.95 in diNAG) as a result of glycosidation of the reducing group is also observed with the methyl glycosides of NAG (Thomas, 1966).

The effect of increasing lysozyme concentrations on the acetamidomethyl proton signals in 0.12M solutions of the glycoside are shown in Fig. 1. Control experiments showed that neither heat-denatured lysozyme or serum albumin could elicit the selective broadening effect. Paper chromatography (using isoamylalcohol:pyridine:water, 1:1:0,8, which separates diNAG from its β-methyl glycoside) showed no trace of diNAG which could have been formed by hydrolysis. Detailed concentration dependence and line width studies were not made due to the limited amounts of glycoside available, but qualitatively the selective broadening of the high field acetamidomethyl

signal is obvious. Inspection of other regions of the spectrum showed no marked changes, in particular no broadening of the methoxyl singlet relative to the low field acetamidomethyl signal was seen.

The simplest conclusion from these preliminary results is that in the binding of this glycoside to lysozyme, the 2-acetamidomethyl group proximal to the methoxyl function interacts more strongly with the binding site than the other acetamidomethyl group. The observed selective broadening of the high field signal may be a reflection of an increase in the relaxation rate of the methyl protons brought about by rotational restriction in the complexed form. The change in relaxation rate is then a result of time averaging between relaxation in free solution and in the complex (Fischer and Jardetzky, 1965).

Some support for this model is available from the X-ray diffraction results (Blake *et al.* 1967), in particular on the lysozyme-chitotriose complex. It is plausible to suppose that the two residues of diNAG β-methylglycoside are bound in the positions of residues "B" and "C" of the trisacharide - the terminal residue "C" corresponding to that disaccharide residue

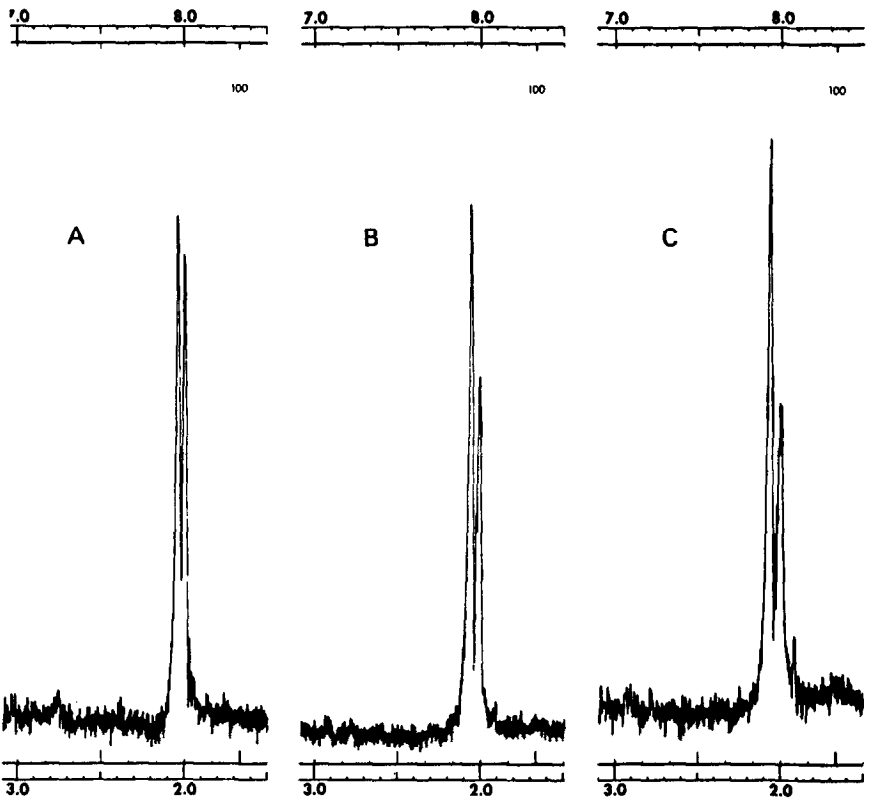

Fig. 1. Acetamidomethyl proton signals in 0.12M solutions of diNAG β-methylglycoside containing 0% w/v (A), 3% (B), and 6% (C) lysozyme.

having the 1-methoxyl group (Professor D. C. Phillips, personal communication). In position C, the acetamidomethyl group is buried deeply in the binding cleft, making contact with Try 108 and Ileu 98. In contrast, the acetamidomethyl group of residue B points away from the enzyme and makes no obvious contacts with it. Restriction of motion of the one acetamidomethyl group resulting in broadening of its PMR signal would then

be expected. Detailed line width studies on this system are in progress in order to evaluate the effect quantitatively.

Acknowledgments

I thank Dr. E. Amundsen for providing facilities for this work, and Professor D. C. Phillips for a discussion and comments on the results.

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