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^{13}C-N.M.R.-SPECTRAL STUDY OF THE BINDING OF Gd^{3+} TO GLYCOPHORIN

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ABSTRACT

Natural-abundance, ^{13}C-n.m.r. spectroscopy was used to study the binding of Gd^{3+} to glycophorin, and also to the tetrasaccharides isolated from glycophorin after treatment of the glycoprotein with NaOH–$NaBH_4$. Gd^{3+} binds to the tetrasaccharide (both in the isolated, reduced form and when still attached to the native glycoprotein), and, especially, to the α-NeuAc residues. In order to cause severe line-broadening of the ^{13}C resonances of α-NeuAc, the ratios of the α-NeuAc residues of glycophorin, and of the isolated, reduced tetrasaccharide, to Gd^{3+} were much higher than that needed for causing similar broadening for 2-*O*-methyl-α-NeuAc–Gd^{3+} solutions. These results indicate that the other carbohydrate residues of the tetrasaccharide may be involved in the binding of Gd^{3+}, producing a stronger metal-ion-binding effect.

INTRODUCTION

The binding of such metal ions as Ca^{2+} to red blood-cells has long been known[1–4], but is still not well understood. Long and Mouat[1] showed that *N*-acetylneuraminic acid (NeuAc), found on red-cell membranes, plays a role in the metal-ion binding.

Because of the possible, cellular importance of the metal ion–NeuAc interactions, we have recently determined[5] the mode of binding of Gd^{3+} and Mn^{2+} to NeuAc in the α-pyranose form**, the anomeric form found in Nature. We showed that both the divalent and trivalent cations bind to α-NeuAc, but in different fashions. The manganese cation can apparently bind at either the head or the tail of α-NeuAc, whereas Gd^{3+} has only one binding mode, "above" the plane of α-NeuAc interacting with the carboxylate anion and the glycerol-1-yl side-chain.

In the red-cell membrane, glycophorin A is the major sialoglycoprotein[6]. Glycophorin A (mol. wt. ~31,000) is the main component of a family of glycophorins that are transmembrane glycoproteins[6–9]; it is composed of 131 amino acids, and

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**Herein, the anomeric designator is used in relation to the D configuration of C-8.

contains a large proportion of carbohydrates (60% by weight[6]), about half of which are α-NeuAc. All of the oligosaccharides are situated near the N-terminal portion of the molecule[6], and are attached to the protein *via* 15 O-linkages (a tetrasaccharide, having the structure given[10], attached to O-3 of L-serine and of L-threonine) and one N-linkage (a complex oligosaccharide attached to N-4 of asparagine-26).

Armitage and co-workers[11,12] have recently shown that Ca^{2+} may interact with the α-NeuAc residues of a glyco-octapeptide derived from glycophorin A. Their studies were, however, limited by the observed small shifts of the carbon resonances (~0.2 p.p.m.) on addition of a two-fold excess (relative to α-NeuAc) of Ca^{2+} ions. Although their work did not determine the exact structure of the Ca^{2+}–α-NeuAc complex, it showed that such metal ions as Ca^{2+} interact (complex) with the oligosaccharides, especially α-NeuAc thereof.

In order to gain some insight into the interactions of metal ions with the oligosaccharides of glycophorin, we decided to use natural-abundance, ^{13}C-n.m.r. spectroscopy to investigate the binding of the paramagnetic relaxation-reagent Gd^{3+} to glycophorin and to the alkali-labile oligosaccharides obtained from glycophorin. Our results conclusively show that Gd^{3+} binds to the tetrasaccharide [both in the isolated, reduced form (**1**) and when it is attached to the native glycoprotein], and, especially, to the α-NeuAc residues. Moreover, the results seem to indicate that the other carbohydrate residues of the tetrasaccharide may also play a role in the metal-ion-binding.

α-NeuAc
2
↓
6
α-NeuAc-(2→3)-β-Gal-(1→3)-GalNAcol
1

EXPERIMENTAL

Materials.—Reagent-grade Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol], lithium diiodosalicylate (LIS), Sephadex-G-15-120, and neuraminidase (2 units) were purchased from Sigma Chemical Co., St. Louis, MO. Gadolinium oxide (99.9%) was obtained from Alfa Products, Danvers, MA. All other chemicals used were of at least reagent-grade quality. Outdated, human blood was the source of the glycophorin and the glycophorin tetrasaccharide used in this study.

Methods. — Carbon-13 n.m.r. spectra were recorded with a JEOL-FX90Q instrument operating at 22.5 MHz (0.21 T; 21.0 kG) in the F.t. mode, as described previously[5]. The preparation of a stock solution of Gd^{3+} (~0.6M) has also been previously described[5]. All chemical shifts are given relative to a trace of internal 1,4-dioxane (added only when chemical shifts were determined), whose chemical shift was taken to be 67.86 p.p.m. downfield from Me_4Si.

Glycophorin was isolated from outdated, human blood according to the LIS–phenol method of Marchesi and Andrews[13]. The final removal of residual lipid, after dialysis, was accomplished by chloroform–methanol extraction as described[14]. The O-linked oligosaccharide release (*via* reduction) was conducted according to the conditions given by Lisowska *et al.*[10], the excess of sodium borohydride being decomposed with acetic acid. The salts and the various oligosaccharides were separated from the modified glycoprotein by ultrafiltration, using an Amicon diaflo equipped with a PM10 membrane. The remaining, modified glycoprotein was dialyzed against distilled water by successive concentrations and redilutions of the glycoprotein in the diaflo cell. All diaflo effluents were pooled, and freeze-dried under diminished pressure at room temperature.

The oligosaccharide–salt mixture was redissolved in distilled H_2O and (after centrifugation to remove a precipitate) fractionated twice on a Sephadex G-15-120 column (187 × 1.7 cm), using distilled water as the eluant. The eluate was monitored at 254 nm by means of a u.v. monitor and by the sialic acid assay method of Warren[15]. The function of the first fractionation was to separate glycophorin, glycophorin fragments, and salts from the oligosaccharides. The second fractionation was used to purify the oligosaccharides further. ^{13}C-N.m.r. spectroscopy, sialic assays, and hexose assays[16] were used to analyze the final oligosaccharide product. No free α-D-NeuAc could be detected in our sample.

Samples for n.m.r. spectroscopy were prepared by dissolving the glycoprotein or oligosaccharide in de-ionized, distilled water. The pH of the samples was maintained between 6 and 7. Addition of Gd^{3+} to the n.m.r. samples was made in μL quantities (total additions ranged from 6 to 30 μL), using an Eppendorf digital pipet.

RESULTS AND DISCUSSION

Fig. 1A shows the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of glycophorin (2mM glycoprotein at neutral pH) at a field strength of 2.1 T (21 kG). Using these protein concentrations and only a 10-mm sample tube, it is impossible to detect single carbon resonances[17]. Therefore, the narrow resonances that we do observe in Fig. 1A result from multiple carbon bands. More specifically, they result from the carbon atoms of[5] α-NeuAc, the preponderant carbohydrate in glycophorin. These resonances can readily be assigned to specific carbon atoms of α-NeuAc by using the ^{13}C-chemical shift data available in the literature[5] for 2-*O*-methyl-α-NeuAc (see Table I); the spectrum in Fig. 1A is essentially identical to other spectra that we had previously obtained[18–20] for homozygous glycophorin A^N.

For several reasons, we decided to investigate the nature of the metal-cation–glycophorin oligosaccharide interactions by using Gd^{3+} as a cation probe. (*1*) Gadolinium mimics lanthanum, which was used in the original, erythrocyte–metal-ion-binding studies by Long and Mouat[1]. They found that trivalent metal-ions bind the strongest to the red-cell membrane. (*2*) Gadolinium is a relaxation probe[21] (line-broadening agent) that will specifically broaden resonances at, or near, the metal-ion binding site. (*3*) We have previously established[5] the mode of interaction of Gd^{3+}

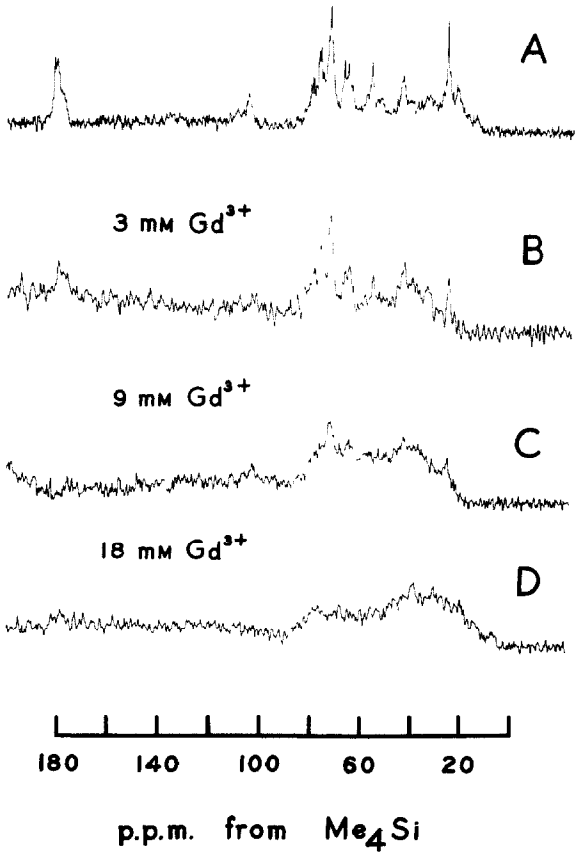

Fig. 1. The effect of Gd^{3+} on the ^{13}C resonances of the proton-decoupled natural-abundance, ^{13}C-n.m.r. spectrum of glycophorin. [All spectra were recorded with a 2-s recycle time and a 5.500 kHz window. The concentration of glycophorin was ~2 mM (in H_2O), pH 6.5. The vertical gain of the spectra containing the paramagnetic relaxation-reagent has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 50,000 accumulations. A line-broadening factor of 1.8 Hz was applied during processing. (B) Sample contained 3mM Gd^{3+}, and required 52,064 accumulations. A line-broadening factor of 3.0 Hz was applied during processing. (C) Sample contained 9mM Gd^{3+}, and required 96,829 accumulations. A line-broadening factor of 4.0 Hz was applied during processing. (D) Sample contained 18mM Gd^{3+}, and required 74,881 accumulations. A line-broadening factor of 4.0 Hz was applied during processing.]

with α-NeuAc; we found that Gd^{3+} binds to α-NeuAc "above" the pyranoid ring, interacting with the carboxylate group and the glycerol-1-yl side-chain.

Figs. 1B, 1C, and 1D depict the effect of addition of Gd^{3+} on the ^{13}C-n.m.r. spectrum of glycophorin. Because the carbon resonances of α-NeuAc are only primarily discernible, we can, therefore, only make judgments concerning the interaction of Gd^{3+} with the α-NeuAc residues of the tetrasaccharides of glycophorin, and not the interaction of Gd^{3+} with the entire, tetrasaccharide moiety. A first glance at the spectra in Fig. 1 conclusively shows that there is a definite interaction between

TABLE I

^{13}C-N.M.R. CHEMICAL-SHIFT DATA FOR 2-*O*-METHYL-α-NeuAc[a]

Carbon atom	*Chemical shift*
α*p*1	174.7
α*p*2	102.0
α*p*3	41.4
α*p*4	69.7[b]
α*p*5	53.3
α*p*6	73.9
α*p*7	69.7[b]
α*p*8	73.1
α*p*9	64.1
-CH_3 (2-*O*-Me)	52.9
-CH_3 (Ac)	23.5
C=O (Ac)	176.4

[a]Data obtained from reference 5. [b]Overlap of resonances.

Gd^{3+} and the α-NeuAc residues; a large number of carbon resonances attributable to α-NeuAc are broadened.

Because of the low signal-to-noise ratio in Fig. 1, and the possibility that multiple, metal-ion-binding sites may exist on α-NeuAc (due to the interaction of the metal ion with all of the carbohydrates of the tetrasaccharide, and not just α-NeuAc)[22], the mode of binding of Gd^{3+} with the carbohydrate residues (including α-NeuAc)

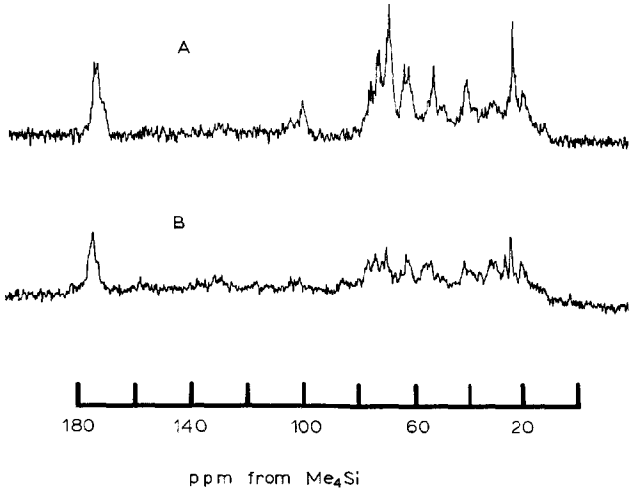

Fig. 2. Proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectra of native and NaOH–$NaBH_4$-treated glycophorin. [Spectra were recorded with 2-s recycle time and a 5.500 kHz window. (A) Same as in Fig. 1A. (B) About 2.5mM glycoprotein in H_2O, pH 7.2. Spectrum required 30,000 accumulations. A line-broadening factor of 2.2 Hz was applied during processing.]

of glycophorin cannot be clearly established. However, it may be concluded that Gd^{3+} does specifically interact with α-NeuAc, and, probably, with the oligosaccharides of glycophorin. In Fig. 1D, where all of the resonances of glycophorin are severely broadened, the ratio of glycophorin to Gd^{3+} is ~1:7; however, the ratio of the α-NeuAc of glycophorin to Gd^{3+} is ~4:1. We have previously observed a lesser degree of line broadening for the carbon-resonances of 2-*O*-methyl-α-D-NeuAc, where the relative ratio of Gd^{3+} in the sample was[5] similarly 4:1.

In order to investigate the nature of the oligosaccharide–Gd^{3+} interactions, we decided to isolate the tetrasaccharides (O-linked to L-Ser and L-Thr) from glycophorin by the β-elimination-reduction reaction ($NaOH–NaBH_4$); this reaction releases the O-linked oligosaccharides with reduction of the carbohydrate that had been attached to the protein. The conditions used in our preparations were those used by Lisowska *et al.*[10] for isolation of the tetrasaccharide from glycophorin; these should prohibit the removal of N-linked carbohydrate chains[23] and prevent the so-called peeling reaction[24,25]. It was to be expected that the major product would be the tetrasaccharide in question; however, minor fragments (and decomposition products) are bound to occur, for several reasons.

Fig. 2B shows the ^{13}C-n.m.r. spectrum of recovered glycophorin that had been treated with $NaOH–NaBH_4$ (see Methods). For comparison, the spectrum of intact glycophorin is shown in Fig. 2A. The spectrum of modified glycophorin (relative to intact glycophorin) indicates that a large proportion of the carbohydrate residues had been removed (especially α-NeuAc). Theoretically, all 15 of the O-linked oligosaccharides should have been cleaved from the glycoprotein. Assays on the modified glycophorin for *N*-acetylneuraminic acid indicated that NeuAc constituted only 2.7% (by weight) of the remaining glycoprotein. This is close to the value to be expected if all 15 of the O-linked tetrasaccharides are cleaved, and only the N-linked, complex oligosaccharide (containing ~2 residues of α-NeuAc) remains. This lessened percentage of α-NeuAc present is in contrast to the expected value of ~25% for glycophorin[6,9,10].

It is known that the conditions used for releasing the oligosaccharides O-linked to L-serine and L-threonine also modify these amino acids[25]; serine is converted into alanine, and threonine into 2-aminobutanoic acid[25]. Therefore, it is possible to determine the number of oligosaccharides that are O-linked to Ser and Thr by determining the decrease in the amounts of serine and threonine that are measured by amino acid analysis after treatment of the glycoprotein with $NaOH–NaBH_4$. Amino acid analysis of the modified glycophorin sample (whose ^{13}C-n.m.r. spectrum is shown in Fig. 2B), relative to that of the unmodified sample, indicated that the modified sample had lost 7 residues of serine and 7 residues of threonine; this is almost identical to the value expected if all 15 of the O-linked oligosaccharides are removed. All of the "wet-chemistry" evidence seems to indicate that the sample, whose ^{13}C spectrum is shown in Fig. 2B, is, indeed, glycophorin that has had all 15 O-linked tetrasaccharides removed.

The carbohydrates (mainly tetrasaccharide) were recovered, and purified, as

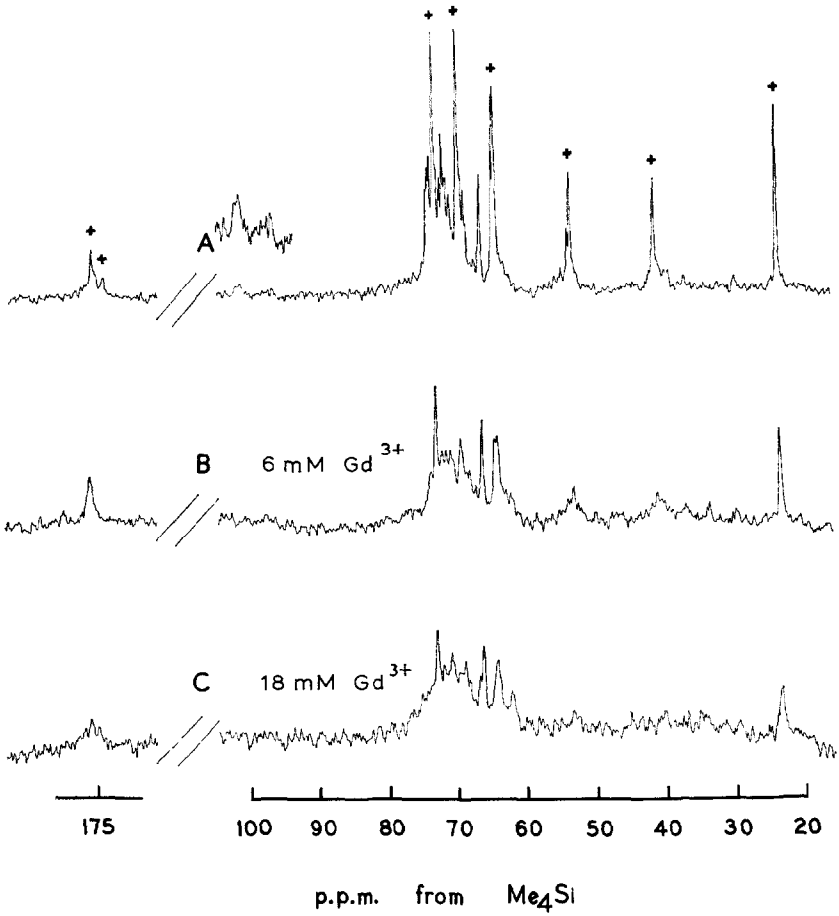

Fig. 3. The effect of Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of the tetrasaccharide isolated from glycophorin. [All spectra were recorded with a 2-s recycle time and a 5.500 kHz window. The concentration of the tetrasaccharide was 32mM (in H_2O), pH 7.0. The vertical gain of the spectra containing the paramagnetic relaxation-reagents has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 50,000 accumulations. A line-broadening factor of 2.8 Hz was applied during processing. (B) Sample contained 6mM Gd^{3+}, and required 63,577 accumulations. A line-broadening factor of 3.6 Hz was applied during processing. (C) Sample contained 18mM Gd^{3+}, and required 50,987 accumulations. A line-broadening factor of 3.9 Hz was applied during processing.]

described in Methods. The ^{13}C-n.m.r. spectrum of the recovered oligosaccharides is shown in Fig. 3A. It is realized that the oligosaccharides present are not entirely in the tetrasaccharide form, and that there may be some carbohydrate decomposition-products present (there are some peaks that occur at ~130 p.p.m.). Moreover, our assay ratio of α-NeuAc relative to Gal (*via* a hexose assay) gave a result of 2:0.8, which is slightly lower than the expected value (2:1).

The proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of the tetrasaccharide, depicted in Fig. 3A, has some broad resonances. Note, for instance,

the anomeric region, and also the region of 76–81 p.p.m., where one would expect to find the signals of the linkage carbon atoms of β-Gal*p* (*βp*3) and α-GalNAc (ol) (C-3), and also the resonances for C-5 of β-Gal*p*. Most of these regions are sharpened considerably, either when the sample is brought to extremely low pH (~1.0) or after it has been treated with neuraminidase in order to release NeuAc. Even in these cases, the signal of the anomeric carbon atom of Gal was difficult to observe.

The markings on top of some of the peaks in Fig. 3A indicate resonances that are attributable to carbon atoms of α-NeuAc (see Table I). The broad resonance of the anomeric carbon atom, *αp*2, is not marked. Also, the peak at ~23 p.p.m. can be attributed to an overlap of two methyl groups, that of the acetyl group of α-NeuAc and the methyl of the acetyl group of GalNAc (ol). Although no attempt has been made to assign all of the peaks in these spectra (due to complexity, the broadness of some resonances, and the fact that some reaction-byproducts may contribute), there are several resonances that indicate that, for the most part, the tetrasaccharide in question has been isolated.

The NeuAc in the tetrasaccharide is in the α-pyranose form, although, were the NeuAc free, it would exist mainly in the β-pyranose form[26]. The marked resonances in Fig. 3A can readily be assigned to specific carbon atoms of α-NeuAc by using the data from the model compound given in Table I. In our spectra, there must be an overlap of the signals of *αp*6 and *αp*8 (at ~72 p.p.m.) and also an overlap of those of *αp*4 and *αp*7 at 69.7 p.p.m. If free β anomer were present, little intensity in the region of 69.7 p.p.m. would be expected, but an increase in intensity[27,28] at 68 p.p.m. Moreover, the resonance for the anomeric carbon atom (*αp*2) should shift from ~101 to ~97 p.p.m., and this phenomenon was observed when the sample was treated with neuraminidase. Therefore, it is considered that the NeuAc present in our sample is attached to other carbohydrates, and is in the α-pyranose form.

To prove the existence in our sample of β-Gal and GalNAc (ol) of the tetrasaccharide is a little more difficult. Many of the carbon resonances of these carbohydrate residues[29–31] are expected to be close to, and possibly to overlap, the intense carbon resonances of α-NeuAc. However, under certain conditions, certain resonances are revealed that are indicative of these carbohydrate residues.

At low pH values, several peaks are discernible that can clearly be attributable to some carbon atoms of GalNAcol. At low pH (~1.0), the resonance of *αp*9 of NeuAc (at ~64 p.p.m.) moves downfield ~1 p.p.m., exposing two resonances of equal intensity. These occur at 64.1 and 63.2 p.p.m., respectively. Moreover, another peak of about equal intensity occurs at 53.6 p.p.m. One of the peaks, at 63 or 64 p.p.m., along with the peak at 53.6 p.p.m., may be attributable to C-1 and C-2 of GalNAcol, respectively[31]. The other peak at 63 or 64 p.p.m. may be attributed[31] to *βp*6 or Gal (see later for further evidence). At low pH, the signals of the anomeric carbon atoms of α-NeuAc also become reasonably sharp, and can be located at ~101 and 98 p.p.m., respectively. Moreover, at low pH, many linkage carbon atoms also now become visible in the region of 75–80 p.p.m.

The results of treatment of the sample with neuraminidase provided informa-

tion about the galactose present in the sample. Aside from the peak at ~63–64 p.p.m., peaks are clearly visible at 80 and 76 p.p.m. These can be attributed to the Gal→GalNAcol disaccharide; the resonance at 76 p.p.m. can be assigned to $\beta p5$ of Gal, and that at 80 p.p.m., to C-3 of glycosylated GalNAcol.

The facts that (*a*) spectra were obtained that already had broadened features, along with possible impurities, and (*b*) all resonances were not specifically assigned make rather difficult the task of obtaining the mode of binding of Gd^{3+} to the tetrasaccharide of glycophorin. On addition of Gd^{3+} (to a concentration of 6mM) to the tetrasaccharide (see Fig. 3B), a number of resonances of α-NeuAc are almost immediately, and entirely, broadened; these are those of $\alpha p6$, $\alpha p8$, $\alpha p7$, $\alpha p4$, $\alpha p3$, $\alpha p5$, $\alpha p1$, and probably, $\alpha p2$ (although it was already broad to start with). This corresponds to a tetrasaccharide:Gd^{3+} ratio of 5:1, or a ratio of α-NeuAc:Gd^{3+} of 10:1. The signals of the carbonyl functional group and the methyl of the acetamido group and, to some extent, that of $\alpha p9$ of NeuAc are not broadened completely. The signals of the carbonyl and methyl groups of the acetamido group also result from the GalNAcol. A prominent peak at 73 p.p.m. in Fig. 3B does not come from the $\alpha p6$, $\alpha p8$ overlap, but from one of the resonances next to it. After the addition of considerably more Gd^{3+}, most of the resonances are broadened beyond recognition, but the resonances of the carbonyl carbon atom and methyl carbon atom of the acetamido group of α-NeuAc or GalNAcol, or both, apparently still remain. Moreover, several peaks remain in the region of 65–75 p.p.m. Interestingly, a ratio of α-NeuAc (of the tetrasaccharide):Gd^{3+} of only 10:1 was needed in order to broaden, almost entirely, the ^{13}C resonances of α-NeuAc. This is in contrast to the much lower ratio of 2-*O*-methyl-α-NeuAc to Gd^{3+} (~4:1) needed to broaden the ^{13}C resonances of 2-*O*-methyl-α-NeuAc. This result indicates that the other carbohydrate residues of the tetrasaccharide may play a role in the metal-ion-binding phenomenon.

The foregoing results indicate that (*i*) Gd^{3+} does bind to α-NeuAc of the tetrasaccharide of glycophorin, (*ii*) the other carbohydrate residues are probably involved to a lesser degree, making α-NeuAc essential for metal-ion-binding[1], and (*iii*) the acetamido group of α-NeuAc may, or may not, be involved in metal-ion-binding.

In conclusion, we have shown that Gd^{3+} binds to α-NeuAc of the glycophorin molecule. Moreover, it interacts very strongly with a tetrasaccharide isolated from glycophorin. Because of the difficulties discussed in the text, we could not determine the exact mode of binding of Gd^{3+} to the tetrasaccharide.

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