Calcium Ion Binding of Three Different Types of Oligo/Polysialic Acids As Studied by Equilibrium Dialysis and Circular Dichroic Methods†

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ABSTRACT: Ca^{2+} binding properties of three different types of oligo/polysialic acid chains, i.e., oligo/poly(Neu5Ac), oligo/poly(Neu5Gc), and oligo/poly(KDN), were studied by equilibrium dialysis and circular dichroism. Colominic acid, high molecular weight polysialoglycoprotein (H-PSGP), low molecular weight polysialoglycoprotein (L-PSGP), and 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN) glycoprotein were found to bind calcium ions with about 8–100 times the affinity of sialic acid monomer. Analysis by equilibrium dialysis of the binding of Ca^{2+} to colominic acid was biphasic, and the high-affinity interaction was shown to change with the degree of polymerization. Specific binding of Ca^{2+} to polysialic acid (polySia) caused characteristic effects in the circular dichroism spectrum. A pronounced decrease in the circular dichroism of polySia at 205 nm was observed upon addition of calcium. H-PSGP was found to bind calcium ions with 3-fold higher affinity than L-PSGP.

α-2→8-Ketosidically linked chains of Neu5Ac,[1] Neu5Gc, and KDN occur as constituents of oligo/polySia (Figure 1; Inoue, 1993; Sato et al., 1993; Troy, 1992). Macromolecules having these oligo/polySia chains were identified as capsular polysaccharides (Sarff et al., 1975; Troy, 1992) of some pathogenic bacteria such as *Neisseria meningitidis* and *Escherichia coli* K1, fish egg polysialoglycoproteins (Inoue & Iwasaki, 1978, 1980; Inoue & Matsumura, 1979, 1980; Inoue et al., 1988; Kanamori et al., 1989, 1990), neural cell adhesion molecules (N-CAM) (Finne et al., 1983; Lackie et al., 1990, 1991; Roth et al., 1987), and sodium channels (James & Agnew, 1987; Zuber et al., 1992). PolySia is also expressed in the embryonic nervous system of *Drosophila melanogaster* (Roth et al., 1992) and on various human tumor cells (Livingston et al., 1988; Moolenaar et al., 1990; Roth et al., 1988). The functional importance of polysialylated macromolecules may be inferred from their presence in a wide variety of phyla from bacteria to higher vertebrates. The polysialic acids (polySia) are often spatiotemporally regulated and only expressed during a limited period of early development (Acheson et al., 1991; Edelman, 1985; Roth et al., 1987, 1992; Rutishauser, 1989). For example, the polySia moiety on N-CAM expressed in embryonic animal brains has been considered to negatively regulate the homophilic interactions between neural cells through a vast, voluminous hydration sphere and the polyanionic nature of the polySia structure (Acheson et al., 1991; Rutishauser, 1989; Yang et al., 1992).

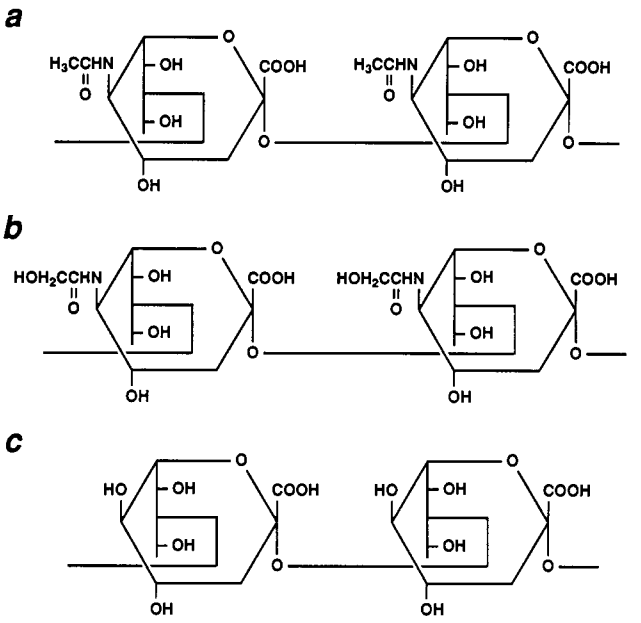

FIGURE 1: Structure of α-2→8-linked disialyl residues in oligo/polySia: (*a*) $(\rightarrow 8Neu5Ac\alpha 2\rightarrow)_n$, (*b*) $(\rightarrow 8Neu5Gc\alpha 2\rightarrow)_n$, and (*c*) $(\rightarrow 8KDN\alpha 2\rightarrow)_n$.

Sialic acid (Neu5Ac) residues were first suggested to be involved in the calcium ion binding phenomenon (Long & Mouat, 1971). Subsequent studies have appeared in the literature concerning the binding of Ca^{2+} to free β-Neu5Ac and bound α-Neu5Ac (Behr & Lehn, 1972, 1973; Czarniecki & Thornton, 1977; Jaques et al., 1977). Strong calcium ion binding (with higher K_a) was observed for β-Neu5Ac, but Ca^{2+} binding with α-Neu5Ac was found to be weak (lower K_a; Czarniecki & Thornton, 1977). In nature, sialic acid residues are present in the α-pyranose form (Sharon, 1975; Tuppy & Gottschalk, 1972). ^{13}C NMR spectroscopy was applied to investigate the binding of Ca^{2+} and paramagnetic relaxation reagents (Mn^{2+} and Gd^{3+}) to derivatives of α-Neu5Ac that mimic α-Neu5Ac in glycoconjugates (Daman

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[1] Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; KDN, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid; NMR, nuclear magnetic resonance; CD, circular dichroism; KDN-gp, KDN-rich glycoprotein; H-PSGP, high molecular weight PSGP; L-PSGP, low molecular weight PSGP; oligo/polySia, oligo/polysialic acid; H-CA, high molecular weight colominic acid; M-CA, medium molecular weight colominic acid; L-CA, low molecular weight colominic acid; st-H-PSGP, sialidase-treated H-PSGP; st-L-PSGP, sialidase-treated L-PSGP; DP, degree of polymerization; K_a, association constant.

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& Dill, 1982). In some cell–cell interactions and recognition reactions, Ca^{2+} present at the cell surfaces is recognized to be essential (Campbell, 1983).

The purpose of our study was to extend our knowledge on the binding of Ca^{2+} to Neu5Ac and to study the interactions of Ca^{2+} with three different types of α-2→8-linked oligo/polySia groups. In this paper we report the use of equilibrium dialysis and circular dichroism (CD) to investigate the stoichiometry and binding strength of oligo/polySia–Ca^{2+} interaction. We also used colominic acid (α-2→8-linked homopolymer of Neu5Ac), H- and L-PSGP containing α-2→8-linked oligo/polyNeu5Gc, which were obtained from rainbow trout eggs, and KDN-glycoproteins bearing α-2→8-linked oligoKDN chains, which was isolated from the ovarian fluid of rainbow trout.

EXPERIMENTAL PROCEDURES

Colominic Acid, H-PSGP, L-PSGP, and KDN-gp. A commercial preparation of colominic acid (Nacalai Tesque, Kyoto) was subjected to Sephacryl S-200 chromatography (2.3 × 160-cm column; equilibrated and eluted with 0.1 M NaCl), and 6-mL fractions were collected. This allowed separation of colominic acid on the basis of its molecular size: Species having high molecular weight (denoted as H-CA) and medium molecular weight (M-CA) were eluted at fractions 42–59 and fractions 60–70, respectively. Both were dialyzed and freeze-dried. A low molecular weight form (L-CA) was prepared by collecting and lyophilizing the material eluted at fractions 72–84 when colominic acid was first treated with 0.01 N HCl at 80 °C for 10 min (Nomoto et al., 1982) and then chromatographed on the same column of Sephacryl S-200. The average degree of polymerization was determined for these three colominic acid fractions by estimating the amounts of reducing terminal Neu5Ac residues and the total amounts of Neu5Ac by the thiobarbituric acid (TBA) (Aminoff, 1961; Uchida et al., 1977) and resorcinol methods (Svennerholm, 1963), respectively. The color development in the TBA method was standardized by using Neu5Ac trimer and tetramer as reference. Three different fractions of colominic acid thus obtained were used for studies by equilibrium dialysis and circular dichroism measurements.

H-PSGP and L-PSGP were purified from the unfertilized and fertilized eggs, respectively, of rainbow trout by the methods previously reported (Inoue & Inoue, 1986; Inoue & Matumura, 1979; Iwasaki et al., 1990; Shimamura et al., 1983). KDN-gp was isolated and purified from the ovarian fluid of rainbow trout by a procedure previously described (Kanamori et al., 1989).

Preparation of Sialidase-Treated H-PSGP (st-H-PSGP) and Sialidase-Treated L-PSGP (st-L-PSGP). st-H- and st-L-PSGP were prepared by removing the oligo/polysialyl chains from H- and L-PSGP (Kitajima et al., 1986, 1988a): In brief, 30 mg of H-PSGP was treated with 4 mL of 50 mM acetic acid buffer (pH 4.0) at 37 °C for 24 h to nonenzymatically remove the bulk of sialic acid residues (Kitazume et al., 1992). The hydrolysate was chromatographed on a column (2.3 × 160 cm; equilibrated and eluted with 0.1 M NaCl/5 mM Tris-HCl, pH 8.0) of Sephacryl S-200 to separate the partially desialylated H-PSGP released from oligoSia. The partially desialylated H-PSGP was concentrated, dissolved in 4 mL of 50 mM acetic acid buffer (pH 4.7), and digested thoroughly with *Arthrobacter ureafaciens* exo-sialidase (Nacalai Tesque, Kyoto) by adding 0.125–0.5 unit at intervals of 5–6 h (total 1.25 units) and incubating at 37 °C for 4 days. After prolonged incubation, the digests were chromatographed on the same column of Sephacryl S-200 to separate from them free Neu5Gc released, and 9.8 mg of the sialidase-treated H-PSGP (st-H-PSGP) was obtained. Ten milligrams of st-L-PSGP was prepared in a similar manner starting from 30 mg of L-PSGP.

KDN-depleted KDN-gp prepared by periodate Smith degradation (Kanamori et al., 1990) was obtained from Dr. A. Kanamori.

Colorimetric Analysis of Carbohydrates. Sialic acid was quantitated by the resorcinol method (Svennerholm, 1963). Neutral sugars were estimated by the phenol–sulfuric acid method (Dubois et al., 1956). KDN was estimated by the method of Kitajima et al. (1992) without prior mild hydrolysis.

Equilibrium Dialysis. Extensive washing of glassware and dialysis tubing, preparation of a series of solutions, and experimental and analytical procedures essentially followed those described by Potter et al. (1983). One-milliliter portions of aqueous solutions of colominic acid (0.7 mg/mL) and glycoproteins (1.0 mg/mL each) were first dialyzed against 10 mM imidazole hydrochloride (pH 7.0) in the presence or absence of 110 mM NaCl and then dialyzed against 10 mM imidazole hydrochloride (pH 7.0) containing 0.03–10 mM $CaCl_2$ and 37–148 kBq of $^{45}CaCl_2$ (1.01 GBq/mg, NEN) with various concentrations of NaCl (from 0 to 150 mM) in the presence or absence of 1 mM $MgCl_2$ at 4 °C. After 24 h, samples were taken from solutions on both sides of the dialysis membrane for liquid scintillation counting. ^{45}Ca dpm values were converted to concentration values by counting aliquots of $CaCl_2/^{45}Ca$ stock solutions. Concentrations of colominic acid or glycoproteins inside the dialysis tubing were determined by specific colorimetric methods. The ratios of bound Ca^{2+} and unbound Ca^{2+} were then calculated. To perform quantitative analysis of binding of Ca^{2+} to polyanionic molecules, it was necessary to correct apparent binding data for the Gibbs–Donnan effect. This was done by measuring the exclusion of the divalent anion sulfate ($Na_2{}^{35}SO_4$, 2.63 GBq/mmol, Amersham) and correcting the bound ^{45}Ca concentration according to the method of Hunter et al. (1988).

Circular Dichroism. Lyophilized samples of colominic acid and glycoproteins were dissolved in 10 mM Tris-HCl (pH 7.3) in the presence or absence of 110 mM NaCl and dialyzed twice against 1 L of the same solution. Concentrations of the high molecular weight ligands (colominic acid and glycoproteins) were determined by the specific colorimetric method. Stock solutions of different concentrations of Ca^{2+}, Mg^{2+}, and Na^+ were prepared, and varying aliquots of these solutions were mixed to give variable concentrations of $Ca^{2+}/Mg^{2+}/Na^+$, to which colominic acid or glycoproteins were added. CD measurements were obtained on a Jasco Model J-600 spectropolarimeter using a quartz cell with a path length of 1 mm for these solutions at room temperature (~20 °C). Results are expressed as mean residue (Neu5Ac, Neu5Gc, and KDN residue for colominic acid, PSGPs, and KDN-gp, respectively) molar ellipticity. By comparison of the CD data for H-PSGP with those for L-PSGP, the molar ellipticity values were converted to those based on the repeating unit, i.e., L-PSGP (mol wt: 9000). The limiting values of molar ellipticity at a divalent cation concentration $[M^{2+}] = 0$ and at a saturating concentration of M^{2+}, where all the binding sites are occupied by M^{2+}, were determined and are denoted as $[\theta]_0$ and $[\theta]_\infty$, respectively. Setting $Y = ([\theta]_0 - [\theta])/([\theta] - [\theta]_\infty)$ and assuming that Ca^{2+} ions bind to the number of independent binding sites n per ligand molecule L with the apparent association quotient K_a, we can formulate the

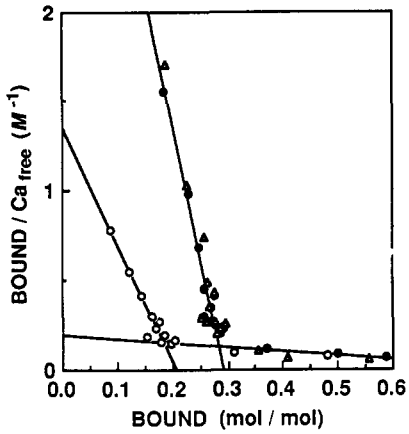

FIGURE 2: Scatchard analysis of calcium binding to colominic acids of different degrees of polymerization by equilibrium dialysis: dialysis temperature, 4 °C; (△) H-CA, (●) M-CA, and (○) L-CA.

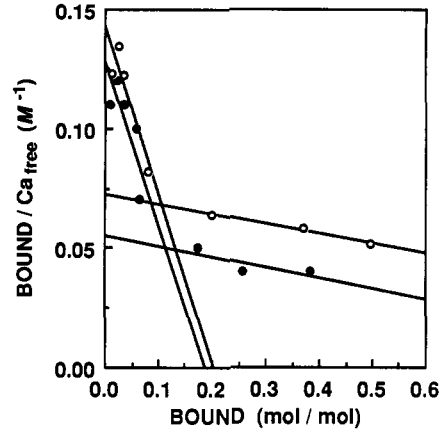

FIGURE 3: Scatchard analysis of calcium binding to colominic acids in the presence of 0.11 M NaCl by equilibrium dialysis: dialysis temperature, 4 °C; (●) M-CA and (○) L-CA.

Table 1: Parameters for Calcium Binding to Colominic Acid

	⟨DP⟩	K_a (M^{-1})	n (mol/mol)
high-affinity binding			
H-CA	24	13.9×10^3	0.30
M-CA	15	14.9×10^3	0.29
L-CA	4.8	6.45×10^3	0.21
low-affinity binding			
H-CA	24	208	0.82
M-CA	15	221	0.88
L-CA	4.8	229	0.80

following equation:

$$Y^2 - [1 + ([Ca^{2+}]_o + 1/K_a)/n[L]_o]Y + [Ca^{2+}]_o/n[L]_o = 0$$

where $[Ca^{2+}]_o$ is the total concentration of Ca^{2+} and $[L]_o$ is the total concentration of ligand L. A two-parameter least-squares fit of all the data points to the above equation yielded the apparent binding constant, K_a, and the number of binding sites, n.

RESULTS

Equilibrium Dialysis. The average DP values for H-CA, M-CA, and L-CA, prepared by the method described, were determined to be 24, 15, and 4.8, respectively. Binding of calcium to these colominic acid samples was studied by equilibrium dialysis using ^{45}Ca as a tracer. The binding of Ca^{2+} to H-CA, M-CA, and L-CA as a function of $[Ca^{2+}]$ is shown by the Scatchard plot in Figure 2. The distinct discontinuity produced in the otherwise linear graphs for each colominic acid that has a different average DP value suggests that two types of Ca^{2+}–colominic acid interaction are taking place. By applying least squares, the Scatchard plots in Figure 2 permit estimates of the apparent binding constants and the number of binding sites for the two distinct processes (Table 1). Both the binding constants (K_a in L/mol) and the number of binding sites (n) for the high-affinity complexes Ca^{2+}–H-CA (K_a, 13.9×10^3; n, 0.30) and Ca^{2+}–M-CA (K_a, 14.9×10^3; n, 0.29) differed from the corresponding values for Ca^{2+}–L-CA (K_a, 6.45×10^3; n, 0.21). The values of n indicated by the intercepts of the high-affinity lines indicate about 1 bound Ca^{2+} per 3 residues of Neu5Ac for H-CA and M-CA, and 1 Ca^{2+} per 5 residues of Neu5Ac for L-CA. The slope of the low affinity lines gave a K_a of about 200 M^{-1} regardless of the DP of the colominic acid used, and the intercept showed 1 bound Ca^{2+} per residue of Neu5Ac.

When the equilibrium dialysis experiments were performed in 0.11 M NaCl, as shown in Figure 3 and Table 2, binding of Ca^{2+} to colominic acid was greatly affected by NaCl and the binding constant was reduced to 1/22 for M-CA and 1/9 for L-CA of the corresponding values for high-affinity binding. In the presence of 0.11 M NaCl, there was no difference in Ca^{2+} binding between M-CA and L-CA; the results indicated a K_a of about 700 M^{-1} and a number of binding sites, n, of 1 Ca^{2+}/$(Neu5Ac)_5$ chain. Binding of Ca^{2+} to colominic acid was inhibited by the increase in ionic strength (Figure 4). When the concentrations of colominic acid and $CaCl_2$ were kept at 0.7 mg/mL and 1 mM, respectively, the extent of Ca^{2+} binding decreased greatly with increasing NaCl concentration and reached 29% of that in the absence of NaCl when the physiological concentration was 110–150 mM. At 1 mM $CaCl_2$, 50% inhibition of Ca^{2+} binding required about 20 mM NaCl, but 65% inhibition was effected by 1 mM $MgCl_2$,

Table 2: Binding of Calcium to Colominic Acid in the Presence of 110 mM NaCl

	K_a (M^{-1})	n (mol/mol)
high-affinity binding		
M-CA	679	0.19
L-CA	708	0.20
low-affinity binding		
M-CA	44.0	1.3
L-CA	40.1	1.8

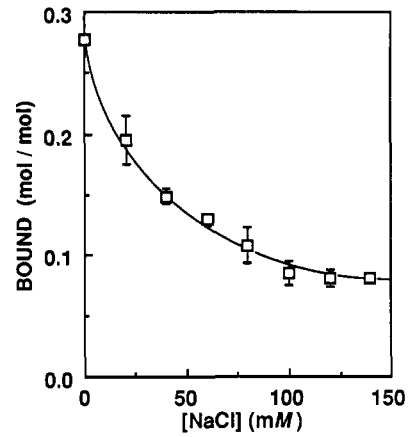

FIGURE 4: Competitive effect of NaCl on Ca^{2+} binding to colominic acid studied by equilibrium dialysis. Calcium binding activity was measured in the presence of varying concentrations of NaCl; dialysis temperature, 4 °C; BOUND refers to the number of Ca^{2+} per Neu5Ac residue (mol/mol). The concentrations of colominic acid and Ca^{2+} were kept constant at 0.7 mg/mL and 1 mM, respectively.

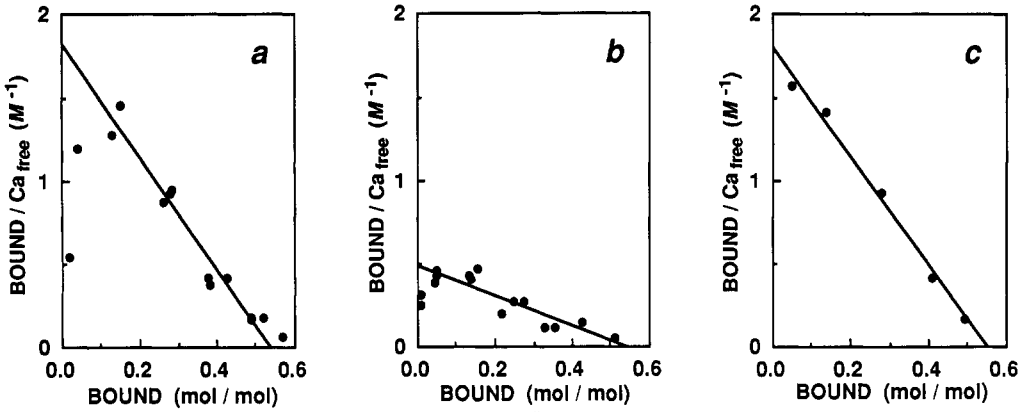

FIGURE 5: Scatchard plots for Ca^{2+} binding to α-2→8-linked oligo/polysialyl group containing glycoproteins and glycopeptide: (*a*) H-PSGP, (*b*) L-PSGP, and (*c*) KDN-gp; dialysis temperature, 4 °C.

Table 3: Association Constant and Number of Binding Sites in the Binding of Calcium to Oligo(poly)sialyl Structures

	H-PSGP	L-PSGP	KDN-gp
K_a (M^{-1})	2.98×10^3	1.00×10^3	2.89×10^3
n (mol/mol)	0.56	0.55	0.59

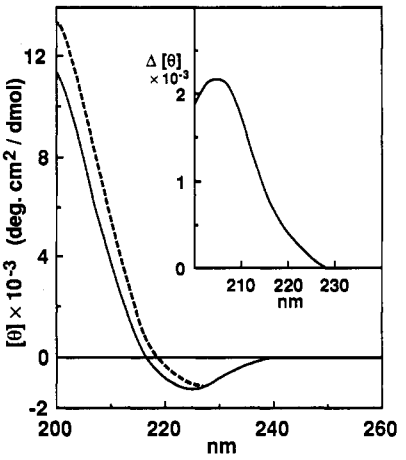

FIGURE 6: Changes in circular dichroic spectra of colominic acid: colominic acid, 0.7 mg/mL, in 10 mM Tris-HCl (pH 7.3) before (- - -) and after (—) addition of Ca^{2+}. Inset: $\Delta[\theta]$ represents the difference between the spectra expressed by the uncomplexed state minus those of the complexed state.

indicating that colominic acid has a much higher affinity for divalent cations than for monovalent cations.

Binding of Ca^{2+} to H-PSGP, L-PSGP, and KDN-gp was examined at low ionic strength by equilibrium dialysis, and the results are shown in Figure 5 and Table 3. The binding constant for L-PSGP (1.00×10^3 M^{-1}) was one-third of that for H-PSGP (2.89×10^3 M^{-1}), whereas the constants for H-PSGP and KDN-gp were essentially identical. These molecules lost their Ca^{2+} binding propensity when oligo/poly(Neu5Gc) and oligo/poly(KDN) chains were removed from PSGPs and KDN-gp, respectively (data not shown).

Circular Dichroism (CD). Ca^{2+}-induced changes in CD spectrum of colominic acid were determined as a function of Ca^{2+} concentration. The CD spectrum of M-CA exhibited a weak negative band at about 225 nm and a stronger positive band at a lower wavelength (Figure 6). The addition of Ca^{2+} to the colominic acid solution did not cause any change of the 225-nm negative band, but did result in a decrease of the lower wavelength positive band. The difference between these spectra was maximal at around 205 nm (Figure 6, inset). Figure 7 shows the Ca^{2+}-induced changes in molar ellipticity

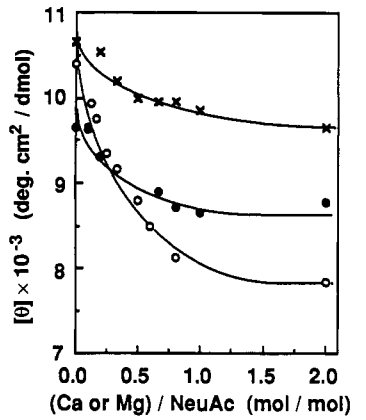

FIGURE 7: Mineral cation induced changes in the CD band of colominic acid (M-CA, 2.3 mM as Neu5Ac) at 205 nm. The CD spectra were recorded as a function of $[Ca^{2+}]$ in the absence of NaCl (○) and in the presence of 0.11 M NaCl (●). The data were also obtained by varying $[Mg^{2+}]$ in the absence of NaCl (×). The ordinate represents molar ellipticity per Neu5Ac residue, and the abscissa represents the ratio of $[Ca^{2+}]$ or $[Mg^{2+}]$ to [Neu5Ac] (mol/mol).

at 205 nm as the Ca^{2+}/Neu5Ac ratio was varied in the buffer solutions when Ca^{2+} was present as the sole mineral cation. The apparent binding constant and the number of binding sites calculated at various degrees of saturation were 10.9×10^3 M^{-1} and 0.39, respectively. When Ca^{2+} was replaced by Mg^{2+}, the Mg^{2+}-induced change in CD at 205 nm was smaller ($\Delta[\theta]_{205} = 1.0 \times 10^3$ deg·cm²/dmol) than the Ca^{2+}-induced change ($\Delta[\theta]_{205} = 2.6 \times 10^3$ deg·cm²/dmol). When the Ca^{2+}-induced changes observed in the presence of 0.11 M NaCl were plotted against Ca^{2+}/Neu5Ac, the results were quite different from those determined in the absence of NaCl (Figure 7). The induced CD changes, measured in varying concentrations of NaCl from 1 to 30 mM in the absence of Ca^{2+}, were negligibly small (not shown). All results indicated that colominic acid has different binding affinities for individual divalent and monovalent cations and that the mineral ion induced change in conformation of colominic acid is also specific to the mineral cations.

H-PSGP, L-PSGP, and KDN-gp all had positive CD bands near 213 nm, and their intensity was reduced upon binding to Ca^{2+} ions (Figure 8). The extent of Ca^{2+} binding was monitored by plotting molar ellipticity per repeating unit of H-PSGP against Ca^{2+}/repeat unit (Figure 9). The change in $[\theta]_{213}$ between zero and saturating levels of Ca^{2+}, when expressed per repeat unit, was greater for H-PSGP ($\Delta[\theta]_{213} = 25 \times 10^3$ deg·cm²/dmol) than for L-PSGP ($\Delta[\theta]_{213} = 6 \times 10^3$ deg·cm²/dmol).

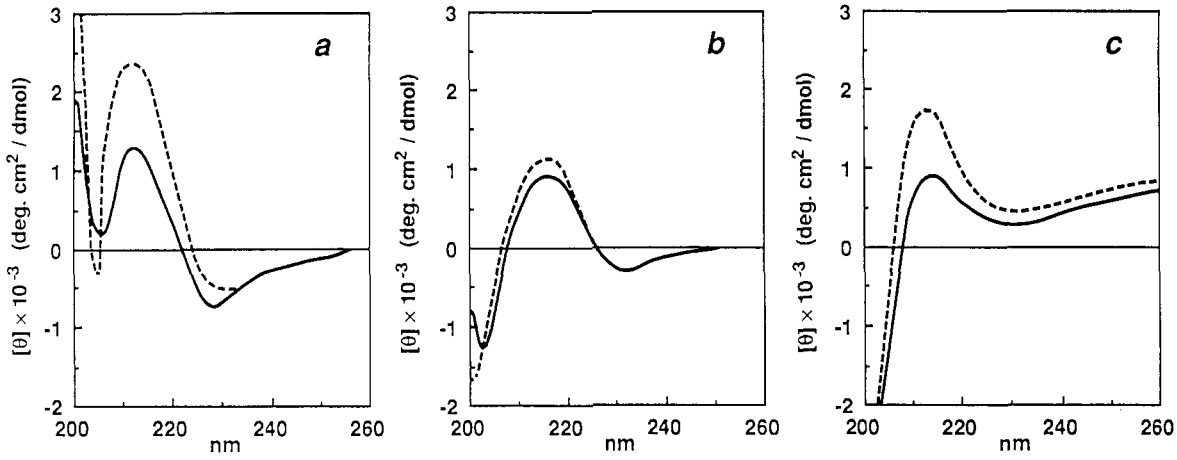

FIGURE 8: CD spectra of (*a*) H-PSGP, (*b*) L-PSGP, and (*c*) KDN-gp, at concentrations of 1 mg/mL in 10 mM Tris-HCl (pH 7.3), showing the effect of Ca^{2+} binding: H-PSGP, L-PSGP, or KDN-gp only (- - -) and H-PSGP, L-PSGP, or KDN-gp plus Ca^{2+}, $[Ca^{2+}]/[Sia] = 2$ mol/mol (—).

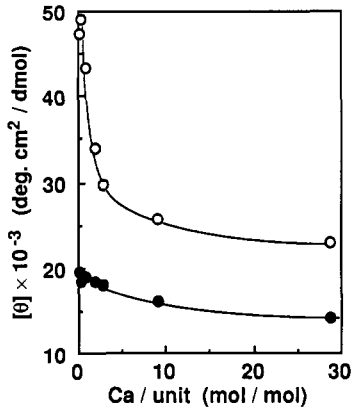

FIGURE 9: Ca^{2+}-induced changes in CD spectra at 213 nm of H-PSGP (○) and L-PSGP (●). The CD data are expressed in terms of molar ellipticity, which for H-PSGP is calculated per repeating unit, and plotted against the concentration ratio of Ca^{2+} to the repeating unit (mol/mol).

DISCUSSION

Binding of Calcium to Poly(Neu5Ac), Colominic Acid. Behr and Lehn (Behr & Lehn, 1972, 1973) were the first to study the complexing of Ca^{2+} with sialic acid. Jaques et al. (1977) studied the binding of Neu5Ac with Ca^{2+} by ^1H and ^{13}C NMR spectroscopy. The NMR titration method allowed us to determine the binding constant, K_a, and the stoichiometry of Ca^{2+} binding to Neu5Ac to be 121 ± 5 M^{-1} and a 1:1 Ca^{2+}–Neu5Ac complex. They also demonstrated that the glycerol side chain of the free sialic acid was intimately involved in the binding of the Ca^{2+} ion. Binding of calcium to the commercially available colominic acid, an α-2→8-linked polymer of Neu5Ac, was studied by ^{13}C NMR measurement, and the colominic acid did not show the large Ca^{2+}-induced changes in the chemical shifts that were observed for Neu5Ac. Since the interketosidic linkages involve C-8 of the glycerol side chain of the Neu5Ac residues, the relatively small changes observed upon addition of Ca^{2+} were interpreted as the glycerol side chains of the Neu5Ac residues that were no longer free to coordinate with Ca^{2+}.

In the present study by equilibrium dialysis using ^{45}Ca as tracer, we found that colominic acid was strongly complexed with Ca^{2+}. When the data on Ca^{2+} binding to colominic acid having $\langle DP \rangle_{av} = 24$ (H-CA) were plotted on Scatchard coordinates, the curve was biphasic, consisting of a distinct discontinuity in the otherwise linear lines. This suggested that two types of Ca^{2+}–colominic acid complexation occurred at different Ca^{2+}/Neu5Ac ratios. From the slopes of the high- and low-affinity lines the apparent Ca^{2+} binding constants were determined to be 13 900 and 208 L/mol. These values are much larger than those for Neu5Ac (121 M^{-1}) (Jaques et al., 1977) and the α-methyl ketoside of Neu5Ac (<2.51 M^{-1}) (Czarniecki & Thornton, 1977). The higher affinity sites of colominic acid had several binding sites for 1 Ca^{2+}/$(Neu5Ac)_3$, indicating that the mode of binding of Ca^{2+} with colominic acid must differ from that with the sialic acid monomer. Although the exact mode of interaction of Ca^{2+} with colominic acid has not yet been established, Ca^{2+} ions may be coordinated both to the anionic atmosphere and to specific sites along the α-2→8-linked poly(Neu5Ac) chains.

The addition of Ca^{2+} to the aqueous solution of M-CA changed the CD spectrum. If we assumed a simple two-state model for describing the Ca^{2+} binding equilibrium of colominic acid, we could estimate the apparent binding constant (K_a) and the number of binding sites (n) by knowing the limiting values of $[\theta]_{free}$ and $[\theta]_{bound}$, which were 10.9×10^3 M^{-1} and 0.39 and agreed satisfactorily with the corresponding values obtained from the high-affinity line (Figure 2) by equilibrium dialysis ($K_a = 14.9 \times 10^3$ M^{-1} and $n = 0.29$). No Ca^{2+}-induced changes in CD were evident for Neu5Ac monomer when Ca^{2+} was added in the range of 0–10 mM (not shown). Thus, the Ca^{2+}-induced CD change observed for colominic acid can not be explained by the simple additive effects of Ca^{2+} ion binding to the component Neu5Ac residues, but may be accommodated by interresidue interactions. From this, we concluded that the Ca^{2+} binding to colominic acid involves a significant change in poly(Neu5Ac) conformation. No Ca^{2+}-induced change in the CD band was observed when the Ca^{2+}/Neu5Ac ratio was varied within the range in which low-affinity binding occurred between colominic acid and Ca^{2+}. The present results thus indicate two types of Ca^{2+}–colominic acid complexing, a strong one that causes characteristic effects in the CD spectrum and a weak one that causes no major change in conformation of the poly(Neu5Ac) chain.

Study of the Ca^{2+} binding of colominic acid that was considered to accompany conformational changes revealed the dependence of K_a and n on the DP of the colominic acid samples used. The values of K_a and n for H-CA ($\langle DP \rangle_{av} = 24$) were almost identical to those for M-CA ($\langle DP \rangle_{av} = 15$) but differed from those for L-CA ($\langle DP \rangle_{av} = 4.8$), indicating a critical chain-length requirement of poly(Neu5Ac) with DP between 15 and 5 for the binding characterized by a K_a of

about 14×10^3 M^{-1} and a n of about 0.30. Most noteworthy are the extensive studies by Jennings and his colleagues (Michon et al., 1987) of the conformational structure of α-2→8-linked poly(Neu5Ac), demonstrating two basic types of linkage conformation: the linkage conformations of the disaccharide residues of the reducing and nonreducing terminal portions are distinctly different from those of the inner continuum of Neu5Ac residues. It should be noted that they carried out experiments only in the absence of Ca^{2+}. It thus follows that a poly(Neu5Ac) of at least seven Neu5Ac residues would be required to accommodate the high-affinity Ca^{2+}–colominic acid complexing.

Ca^{2+} had much higher affinity for colominic acid than Na^+ and Mg^{2+}. Binding of Ca^{2+} to colominic acid was inhibited by an equivalent concentration of Mg^{2+}, but Na^+ had no inhibitory effect. However, at 0.11 M NaCl, Ca^{2+} binding capacity was greatly reduced to 679 from 14.9×10^3 M^{-1} in the absence of NaCl. Thus, oligo/polySia may be considered to be important in regulating the cytosolic and/or extracellular free Ca^{2+} and in regulating cell-surface molecules such as N-CAM through Ca^{2+}-induced structural changes on the oligo/polySia chains. The Ca^{2+} binding property of α-2→8-linked poly(Neu5Ac) may be relevant to a possible functional role of the polySia chain of N-CAM in controlling the extracellular Ca^{2+} concentration. The Ca^{2+}-dependent conformational changes of the poly(Neu5Ac) chain may also induce a change in the N-CAM conformation and consequently regulate cell adhesion (Rutishauser et al., 1988) as well as a possible function of the N-CAM molecule as a signal transducer (Schuch et al., 1989; Doherty et al., 1991).

Binding of Calcium to H-PSGP, L-PSGP, and KDN-gp. Binding of Ca^{2+} to H-PSGP, L-PSGP, and KDN-gp has been studied by equilibrium dialysis. Calcium binding to all of these molecules occurred with apparent binding constants (K_a) of 2.98×10^3, 1.00×10^3, and 2.89×10^3 M^{-1}, respectively, which are higher than the reported binding constants of 121 and 193 M^{-1} for the formation of the 1:1 complexes Ca^{2+}–Neu5Ac (Jaques et al., 1977) and Ca^{2+}–Neu5Gc (Jaques et al., 1980), respectively. Since the removal of oligo/poly(Neu5Gc) and oligo/poly(KDN) from PSGPs and KDN-gp abolished their Ca^{2+} binding capacities, the carboxylate groups of these macromolecules must have been critically involved in the binding of Ca^{2+}. The smaller binding affinities observed for PSGPs and KDN-gp, compared to those for colominic acid (H- and M-CA), are likely due to the smaller average DP values of PSGPs ($\langle DP \rangle_{av} = \sim 6$) (Inoue & Iwasaki, 1980; Kitajima et al., 1986) and KDN-gp ($\langle DP \rangle_{av} = \sim 5$) (Kanamori et al., 1990). The number of sialic acid residues required to bind 1 Ca^{2+} was 5 for L-CA and was estimated to be 2 for H-PSGP, L-PSGP, and KDN-gp. Nothing is known of the molecular mechanisms that underlie PSGP–Ca^{2+} interactions, but the present results indicate that the mode of calcium binding to H- and L-PSGP or KDN-gp differs from colominic acid–Ca^{2+} interactions partly because several oligo/poly(Neu5Gc)- and oligo/poly(KDN)-containing glycan chains are linked to the core proteins through the closely spaced amino acid residues (Inoue & Inoue, 1986; Kanamori et al., 1990; Kitajima et al., 1986).

Difference in Ca^{2+} Binding Affinity to H-PSGP and L-PSGP. H-PSGP (molecular weight, about 200K) is a characteristic constituent of cortical alveoli in the unfertilized eggs of salmonid fish species (Inoue & Inoue, 1986; Inoue et al., 1987; Kitajima et al., 1988b). The 200-kDa PSGP was shown to be made up of tandem repetitions of a glycotridecapeptide unit. In every repeating unit there are three attachment sites for oligo/polysialylglycan chains (Inoue & Inoue, 1986; Kitajima et al., 1986). Upon fertilization H-PSGP was found to undergo rapid proteolytic depolymerization into the repeating unit, or L-PSGP (molecular weight, 8∼9K) (Inoue & Inoue, 1986). In the present study, the effect of the molecular size of PSGP on calcium binding with PSGP was studied by comparison of H- and L-PSGPs which differ only in size.

Ca^{2+} binding affinity for L-PSGP was one-third of that for H-PSGP, as studied by equilibrium dialysis, whereas the number of binding sites (n) did not change on going from H-PSGP to L-PSGP. H-PSGP was more strongly and preferentially complexed with Ca^{2+} ion than L-PSGP, as indicated by induced CD changes particularly at 213 nm. Neither st-H-PSGP nor st-L-PSGP showed any detectable calcium-induced changes (data not shown), indicating that oligo/polysialyl residues are responsible for Ca^{2+} binding of H- and L-PSGPs. However, as described above, the order of Ca^{2+} binding affinities is H-PSGP > L-PSGP and critically depends upon charge density; L-PSGP binds Ca^{2+} with only one-third of the affinity of H-PSGP. These findings have implications for the mechanism of calcium release at fertilization. According to this view, the Ca^{2+} ions bound to H-PSGP represent a reservoir of calcium for liberation at fertilization (upon conversion of H-PSGP to L-PSGP).

One main reason that the function of PSGP is not well known is the difficulty of observing the eggs of rainbow trout under a microscope and cultivating the embryos in the laboratory. A question of interest is the Ca^{2+} concentration within the cortical alveoli. Although there is no information about the amount of Ca^{2+} in a cortical alveolus and it is still difficult to determine it directly, the concentration of Ca^{2+} in cortical alveoli was estimated to be 93 mM on the basis of the average values of egg and cortical alveolus diameters and the amount of H-PSGP present in an egg with a value of Neu5Gc/Ca^{2+} = 2:1. The derived value of $[Ca^{2+}]$ in cortical alveoli of rainbow trout compared well with those of Gillot et al. (Gillot et al., 1991); values for $[Ca^{2+}]$ of 30 and 95 mM were reported for two different species of sea urchin, based on X-ray microanalysis. Upon fertilization salmonid fish eggs were forced to be in an environment of low osmolarity, and water tended to flow into the perivitelline space, causing it to swell (Kobayashi, 1985; Yamamoto, 1961). Rainbow trout eggs increased their weight by about 20% when immersed in water. This allowed us to estimate the perivitelline space fully formed by fertilization to be about 6.7×10^{-3} mL, which consequently led to estimates of Ca^{2+} concentration and Neu5Gc residue concentration of L-PSGP (originally present in the cortical alveoli of unfertilized eggs) within the perivitelline space of 4.7 and 9.4 mM, respectively. It was previously reported that about 0.2% of the Ca^{2+} present in the eggs of dog salmon was released from the egg through the vitelline envelope within 30 min after water activation (Yamamoto, 1979). If this is also the case with the eggs of rainbow trout, approximately 1.9% of the Ca^{2+} ions which were stored within the cortical alveoli were estimated to be released from the eggs at fertilization. Therefore, L-PSGP is considered to function as a reservoir for Ca^{2+} in the perivitelline space surrounding an early embryo.

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