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High affinity binding of long-chain polysialic acid to antibody, and modulation by divalent cations and polyamines

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Abstract

Long-chain polysialic acid (PSA) is expressed on the vertebrate neural cell adhesion molecule (NCAM) during neuronal plasticity. Its structural similarity to the capsular PSAs of some pathogenic bacteria has hampered the development of polysaccharide vaccines against meningitis. The antibodies formed during immunization require a long epitope for binding, and cross-react with host tissue PSA. The nature of the epitope and possible external effectors involved are still unclear. We have evaluated the interaction of PSA with its antibody mAb735 by surface plasmon resonance. The influences of PSA chain length, pH, temperature, ionic environment, and polyamines were also determined.

The antibody binding affinity was found to dramatically increase with PSA chain length. A sub-nanomolar dissociation constant (K_D = 8.5×10^{-10} M) was obtained for the binding of very long chain native MenB polysaccharides (~200 Neu5Ac-residues). Colominic acid from *Escherichia coli* K1 (~100 residues) and shorter polymers exhibited progressively weaker affinities. The antibody also bound tightly ($K_D \sim 5 \times 10^{-9}$ M) to polysialylated glycopeptides from human embryonal brain. The effects of pH and ionic strength suggested that the interaction is largely electrostatic. Ca^{2+} and Mn^{2+} ions promoted the observed surface plasmon resonance response in a concentration dependent fashion. Spermine increased the response in a similar way. Our results suggest that divalent cations and polyamines may play significant role in the regulation of the PSA epitope presentation in vivo.

Keywords: Polysialic acid; Neural cell adhesion molecule; Polyamines; Calcium; Cations; Antibody affinity

1. Introduction

Polysialic acid (PSA) is a α(2–8)-linked homopolymer of *N*-acetylneuraminic acid (Neu5Ac). It is present in the N-glycans of neural cell adhesion molecules (NCAM) of vertebrate cells (Finne et al., 1983a) as well as in capsular polysaccharides of some pathogenic bacteria (Jennings et al., 1984). In nervous tissue, PSA is thought to modulate NCAM-mediated cell adhesion events, such as the axonal guidance in nerve developing and regeneration, as well as the establishing of new neural connections during learning and memory formation (Rutishauser and Landmesser, 1996). NCAM–PSA is present on growth cones of propagating axons and its expression is strictly temporally regulated, peaking in humans at the time of birth (Troy, 1992). The newborn are most susceptible to acute bacterial meningitis and there are currently no preventive vaccines against the two main causative agents, *Escherichia coli* K1 and *Neisseria meningitidis* group B (MenB). The observed poor immunogenicity of these bacteria is suggested to result from tolerance caused by the structural similarity of their polysialic acid capsules to host NCAM–PSA (Finne, 1982; Colino and Outschoorn, 1998; Finne et al., 1983b).

Abbreviations: CD, circular dichroism; ColA, colominic acid; d.p., degree of polymerization; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; FID–GLC, flame ionization detection–gas–liquid chromatography; FPLC, fast protein liquid chromatography; GBS, group B streptococci; GLC–MS, gas–liquid chromatography–mass spectrometry; HA, hyaluronic acid; HBS, HEPES buffered saline; MenB, group B meningococci; NCAM, neural cell adhesion molecule; Neu5Ac, *N*-acetylneuraminic acid; NHS, *N*-hydroxysuccinimide; PSA, polysialic acid; Put, putrescine; RU, response unit; Sp, spermine; Spd, spermidine; SPR, surface plasmon resonance

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We and others (Jennings et al., 1985) have previously found that most antibodies against polysialic acids to require an unusually long epitope of ~10 sialyl residues for ligand binding in solution (Finne and Mäkelä, 1985; Häyrinen et al., 1989, 1995). Our immunoblot experiments together with previous ELISA-studies have suggested a requirement for even longer epitopes in solid phase assays (Tikkanen et al., 1995; Hurpin et al., 1992). For a polymer of 16 residues, a moderate binding affinity ($K_{aff} = 2 \times 10^{-6}$ M) was observed. (Häyrinen et al., 1989). However, the methods (such as ELISA, equilibrium dialysis and microcalorimetry) employed in previous studies could not assess the contributions of the association and dissociation phases to the observed net affinities (Kabat et al., 1986; Diaz-Romero and Outschoorn, 1993; Mandrell and Zollinger, 1987; Wessels et al., 1987). Also, variations in the experimental conditions (temperature, pH, ionic strength and buffer composition) make the comparison of results difficult, since not much is known about their effect on the binding. Although several models for the PSA epitopes have been constructed based on the results obtained by NMR spectroscopy, potential energy calculations, ligand docking studies and X-ray crystallography of the antibody combining site (Michon et al., 1987; Yamasaki and Bacon, 1991; Brisson et al., 1992; Evans et al., 1995), the actual mechanisms of antibody interaction have remained unclear.

PSA is usually considered to act as a repulsive element in NCAM interactions, but recent studies have also reported attractive properties (Storms and Rutishauser, 1998), including ability to form filament bundles (Toikka et al., 1998). Due to their polyanionic nature, PSA epitopes are suggested to employ marked electrostatic components in their binding events (Häyrinen et al., 1989; Evans et al., 1995).

Binding of divalent cations (such as Ca^{2+}) could also affect epitope presentation by inducing conformational changes, or by neutralizing the negative charges of the carboxyl groups (Jaques et al., 1976; Shimoda et al., 1994), although there is also contradictory evidence (Aubin and Prestegard, 1993; Vliegenthart et al., 1982). Two circular dichroism studies have yielded conflicting results on the cationic interactions (Shimoda et al., 1994; Bystricky et al., 1997): Bystricky et al. (1997) observed calcium to change the conformer distribution of colominic acid oligomers until d.p. 9, although, in another study no conformational changes were observed (Shimoda et al., 1994). Possible effects of calcium are intriguing as it is involved in several adhesion-related events in the central nervous system: Ca^{2+} affects the conformation, aggregation and refolding transitions of ependymin (a cell-adhesion related molecule associated in neural plasticity) (Ganss and Hoffmann, 1993). Also Ca^{2+} binding to sialic acids facilitates fibrinogen monomer aggregation in fibrin assembly (Dang et al., 1989) and Ca^{2+} transients were shown to control in vivo regulation of axon extension and growth-cone path finding (Gomez and Spitzer, 1999)—a phenomenon also associated with NCAM–PSA. Although the extra-cellular Ca^{2+} concentration in brain is normally low, local concentrations may differ by several orders of magnitude due to gradients, mirroring intracellular transients (Nicholson, 1980). Ca^{2+}-transients are very sensitive regulation signals triggered by chondroitin sulphate proteoglycans and suppressed by L1 and laminin (Green et al., 1997). Interestingly, L1 is suggested to bind to NCAM, which also acts in synergistic way during development (Doherty et al., 1993). Additionally, Mn^{2+} was recently shown to specifically promote the interaction of L1 cell adhesion molecules with β_1 integrins (Felding-Habermann et al., 1997)—a similar interaction controlled by extra cellular Ca^{2+}.

Polyamines are ubiquitous aliphatic amines found in most living organisms (Tabor and Tabor, 1984). They are enriched in nervous tissue and malignant tumors, and spermine has been also found to bind to the NMDA receptor in the brain (Mantione et al., 1990). Recently increased polyamine levels were shown to be neuro-protective in rat cerebral ischemia (Lukkarinen et al., 1999). The recovery from ischemic stroke requires neuronal plasticity, thus also involving PSA. As these polycationic amines and polyanionic PSA are spatio-temporally co-expressed in the brain, their possible interactions need to be investigated.

In this study, we have probed the interactions of polysialic acid with a mouse monoclonal anti-MenB polysaccharide antibody using surface plasmon resonance (SPR). The technique was recently introduced in the real-time analysis of carbohydrate–protein interactions (MacKenzie et al., 1996; Blikstad et al., 1996) and it allows the simultaneous determination of both the kinetic and steady-state parameters. In addition to the binding properties and affinities of polysaccharides of different chain lengths, the effects of temperature, pH, ionic strength and cations on the antibody binding were studied. The results show that the antibody has progressively higher affinity for large polymers, and that the interaction is influenced by divalent cations and spermine.

2. Materials and methods

2.1. Preparation of poly- and oligosaccharides

Neu5Ac-oligomers of defined chain-lengths were prepared by thermal hydrolysis of colominic acid (Sigma). Aliquots of colominic acid in water (1–5 mg/ml) were kept in boiling water bath or heat block (96 °C) for 20–120 min, depending on polymer sizes required. The resulting oligosaccharides were separated by anion-exchange chromatography on FPLC equipped with a MonoQ HR 5/5 column (Pharmacia AB, Uppsala, Sweden) at +4 °C using a NaCl gradient (0–1.0 M). Samples of 0.5 ml (corresponding to ~0.5 mg of carbohydrate) were applied to the column and 0.5 ml fractions were collected at a flow rate of 1.0 ml/min. The gradient was monitored by conductivity and the carbohydrates were detected by their UV-absorbance at 214 nm. The method was optimized for short chain oligosaccharides,

and baseline separation could be achieved for oligomers up to 16 residues long ($Neu5Ac_{1–16}$). The higher molecular mass fractions not completely resolved, were pooled in batches by increasing average size (and labeled HMW1, HMW2 and HMW3, respectively). Fractions from several chromatographic separations were combined, lyophilized with a slight molar excess of sodium bicarbonate to minimize lactone formation, and stored frozen. The PSA-oligomers were desalted on FPLC using four HiTrap-desalting columns (Pharmacia Biotech, The Netherlands) connected in series and eluted with 5 mM ammonium bicarbonate at a flow rate of 4.0 ml/min. Sialic acids were detected by their UV-absorbance at 214 nm and salts were monitored by conductivity. The desalted samples were lyophilized with sodium bicarbonate (see earlier) and stored frozen. For identification, the oligosaccharides were dissolved in water or D_2O and subsequently analyzed by one- and two-dimensional ^{1}H-NMR spectroscopy at 500 MHz (Bruker AMX500). Native meningococcal group B polysaccharide was obtained from Connaught Laboratories, Swiftwater, PA. The size range of the glycans was determined by gel electrophoresis in 15% polyacrylamide gels (Pelkonen et al., 1988) using marker dyes and Neu5Ac-oligosaccharides of defined chain length as reference compounds. Sialyllactose and disialyllactose were purified from bovine colostrum (Parkkinen and Finne, 1987).

Human embryonal brain glycopeptides were isolated from abortion material (obtained in the University Hospital of Kuopio, Finland, after approval of the Ethical Committee of the University and by the consent of the parents). Delipidation with chloroform/methanol extraction, subsequent proteinase K digestion and precipitation of polyanionic compounds (nucleic acids, glycosaminoglycans) other than PSA by cetylpyridinium chloride, were carried out as previously described (Finne, 1982). Glycopeptides were desalted on a P-2 (Bio-Gel P-2/200–400 mesh, Bio-Rad, Richmond, CA, USA) gel filtration column (75 cm × 2.5 cm, total bed volume 368 ml), with 10 ml of Bio-Gel P-6 DG (Bio-Rad, Richmond, CA, USA) on the bottom to prevent clogging of the lower frit. The column was eluted with 50 mM ammonium bicarbonate, pH 7.0 at the flow rate of 0.5 ml/min and the fractions (12 ml) collected were analyzed colorimetrically for sialic acid (Miettinen and Takki-Luukkainen, 1959). The fractions containing sialylated glycopeptides were pooled, lyophilized with sodium bicarbonate (see earlier) and stored frozen.

Polysialylated glycans were purified by immunoaffinity chromatography on immobilized mouse monoclonal anti-MenB IgG_{2a} anti-polysialic acid antibody mAb735, prepared as previously described (Frosch et al., 1985). A total of 63 mg of mAb735 was coupled to CNBr–Sepharose 4B (Pharmacia Biotech, The Netherlands) according to manufacturer's instructions. A coupling efficiency of 98.5% was achieved (corresponding to ~400 nmol of bound IgG), as determined by monitoring the UV-absorbance at 280 nm of the reaction mixture and subsequent washes. The gel was packed to an 8-ml column and its binding specificity was tested with sialylated oligosaccharides of defined length (see earlier). The human embryonal brain glycopeptide preparation was dissolved in water and passed slowly (0.1 ml/min) over the column, which was subsequently washed with 10 volumes elution buffer. The flow-through and wash solutions were concentrated and desalted by ultrafiltration over a YM3 membrane (Amicon, Danvers, MA, USA) under pressurized nitrogen at +4 °C, and recycled back to the column until no further binding was observed. Since the initial elution experiments with high pH (0.1 M diethylamine/10 mM Tris, pH 10.5) buffer yielded unsatisfactory results, stepwise NaCl gradients (10 mM–1.0 M) were used to elute the bound polysialylated material. After lyophilization and desalting, the presence and sialylation degree of the brain polysialylated glycans were confirmed by FID–GLC, GLC–MS and ^{1}H-NMR. Calculations of the concentration of the glycans were based on a previously suggested trimannosyl core glycan structure (Finne, 1982; Kudo et al., 1996; Nelson et al., 1995; Zamze et al., 1998; von der Ohe et al., 2002).

2.2. Surface plasmon resonance (SPR) measurements

Binding of the various analytes to the mAb735 anti-MenB antibody was determined with a BIA-Core 2000^{TM} apparatus (Pharmacia Biotech, The Netherlands). The antibody was immobilized on two flow cells of a CM5 research grade sensor chip using the standard amino coupling kit at a flow rate of 5 μl/min. Following a 4 min activation of the surface with 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC)/0.05 M *N*-hydroxysuccinimide (NHS), the antibody, at concentrations of 50 and 25 μg/ml, respectively, was coupled to the surface in 10 mM sodium acetate, pH 4.8 for 10 min at RT. Unreacted NHS esters were blocked with 1 M ethanolamine, pH 8.0 (4 min pulse). The individual couplings resulted in 11,800 and 6000 response units (RU) of immobilized ligand per flow-cell corresponding to approximately 10 and 5 ng protein/mm^2, respectively. A mouse monoclonal anti-sheep myoglobin antibody (Pharmacia Biotech, The Netherlands) was used as a control for non-specific binding because of its similar species (mouse), immunoglobulin sub-class (IgG_{2a}) and lack of cross-reactivity with carbohydrate antigens, as specified by the supplier. The control antibody (50 μg/ml) was immobilized on the adjacent vacant flow cell under identical conditions, resulting in 8400 RU of bound ligand. An activated and blocked flow-cell without immobilized ligand was used to evaluate non-specific binding.

Unless otherwise indicated, the measurements were carried out at 25 °C in HBS-buffer (10 mM HEPES/150 mM NaCl/3 mM EDTA/0.005% Surfactant P-20, pH 7.4) supplied by the manufacturer, using a flow rate of 5 μl/min. The effects of pH and various ions were studied in 100 mM sodium acetate, 100 mM Tris–HCl (pH varied from 4.5 to 7.9) or 10 mM imidazole–HCl buffer (pH 7.0). The possible

effects of Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were studied by varying the concentrations of the ions by adding NaCl, $CaCl_2$, $MgCl_2$, $MnCl_2$ or $ZnCl_2$, respectively. The standard HBS-buffer was substituted with acetate or Tris-based buffers to avoid chelating the ions studied by EDTA present in HBS.

Since the commonly employed washing of the surface with 10 mM HCl resulted in only partial regeneration of the flow cell, a new regeneration method was introduced, in which a 4 min (20 μl) pulse of 1 M NaCl was followed by a 2 min pulse (10 μl) of 0.1 M HCl. For all samples, response curves were also recorded on control surfaces. Results were calculated after subtraction of the control values using the BIA Evaluation 3.0 software (Pharmacia Biotech, The Netherlands) and by Scatchard analysis. The equations employed are defined elsewhere (MacKenzie et al., 1996; BIA-Evaluation 3.0 Manual) and the terminology used for defining the various constants is in accordance with Mammen et al. (1998). K_D rather than K_A was used as basis of comparing the affinities.

3. Results

3.1. Characterization of polysialic acid oligo- and polymers

Prior to SPR-measurements the Neu5Ac-polymers were analyzed with ^{1}H-NMR spectroscopy. All major signals in the one-dimensional spectra could be assigned to specific nuclei in the oligosaccharides by two-dimensional techniques (complete assignment will be presented elsewhere), and the values obtained for chemical shifts (δ) were in accordance with those previously published for similar compounds (Michon et al., 1987). The homogeneity of preparations and absence of contaminants were determined by gel electrophoresis (Pelkonen et al., 1988), NMR and ion-exchange chromatography. Shorter oligomer preparations (d.p. 1–7) contained <5% and longer polymers (d.p. 8–16) approximately 10% of each adjacent oligomer. The degrees of polymerization were determined by NMR from the ratios of axial and equatorial pyranose ring H-3 proton signals resulting from terminal ('A' and 'C') and internal ('B') Neu5Ac-residues (Table 1). Colominic acid had a very wide size distribution with average maximum at 100 (used also as d.p. in concentration calculations), which was in agreement with the average maximum of 80–120 residues previously determined by gel electrophoresis (Pelkonen et al., 1988). For the MenB polysaccharide, the average degree of polymerization was previously determined to be 150–300 (200-mer used in concentration calculations) by gel electrophoresis (Pelkonen et al., 1988), due to low signal intensity and line broadening preventing its analysis by NMR.

The polysialylated nature of PSA-glycopeptides isolated from human embryonal brain was confirmed by ^{1}H-NMR spectroscopy. In addition to strong Neu5Ac signals, mannose, galactose, fucose and *N*-acetylglucosamine were also detected (complete structure will be described elsewhere). Following methanolysis, monosaccharide analyses by FID–GLC and GLC–MS suggested the presence of partially fucosylated complex-type glycans with ≥60 Neu5Ac residues per glycan. This is close to the sialylation degrees reported previously for chicken and brain NCAM–PSA (54 residues), neuroblastoma cell line PSA (58 residues) and polysialyltransferase-transfected HeLa-cells (50 residues) (Kudo et al., 1996; Livingston et al., 1988; Angata et al., 1998).

Table 1
^{1}H-NMR-analysis of oligo- and polysialic acids: determination of degree of polymerization from intensities of signals originating from reducing end (A) non-reducing end (C) and internal (B) residues

Compound[a]	Relative intensity[b]				d.p.
	H-3eq		H-3ax		
	$C_{2.76\,ppm}$	$B_{2.70\,ppm}$	$A_{2.22\,ppm}$	$ABC_{1.72\,ppm}$	
$Neu5Ac_1$	–	–	1.00	1.00	1
$Neu5Ac_2$	1.02	–	1.00	2.01	2
$Neu5Ac_3$	0.98	2.10	1.00	3.11	3
$Neu5Ac_7$	1.24	5.40	1.00	7.08	7
$Neu5Ac_{16}$	1.06	14.30	1.00	15.70	16
HMW1	1.10	29.10	1.00	31.10	∼31[c]
HMW2	1.00	51.30	1.00	50.40	∼50[c]
ColA	n.d.[d]	∼100	1.00	∼100	∼100[c]

[a] Data for $Neu5Ac_{4–6}$ and $Neu5Ac_{7–15}$not shown.
[b] Calculated relative to $A_{2.22\,ppm}$.
[c] Average d.p. of a mixture.
[d] Not determined due to low signal-to-noise ratio.

3.2. Polysaccharide chain length requirement of antibody binding

To study the PSA–antibody interaction, samples of Neu5Ac-oligomers of defined lengths (see Section 2) were injected over the antibodies immobilized on sensor chips of the surface plasmon resonance apparatus. The obtained response curves, or 'sensorgrams' (Fig. 1) were used to evaluate the relative amounts of oligosaccharide binding, the kinetics of the interaction, and the steady-state binding constants. As compared to proteins and peptides of corresponding molecular masses, constantly lower responses were observed for the polysialic acid samples, due to their lower refractive indexes typical for carbohydrates. No marked baseline drifting or delays in the response due to limited mass transfer were observed. The results obtained using a flow cell with lower ligand density (6000 RU) were comparable.

The relative maximum binding values (Table 2) were determined at fixed equimolar concentrations, since the high concentrations of oligosaccharides needed for saturation were observed to inhibit the interaction. No binding to oligomers shorter than d.p. 10 could be detected, in

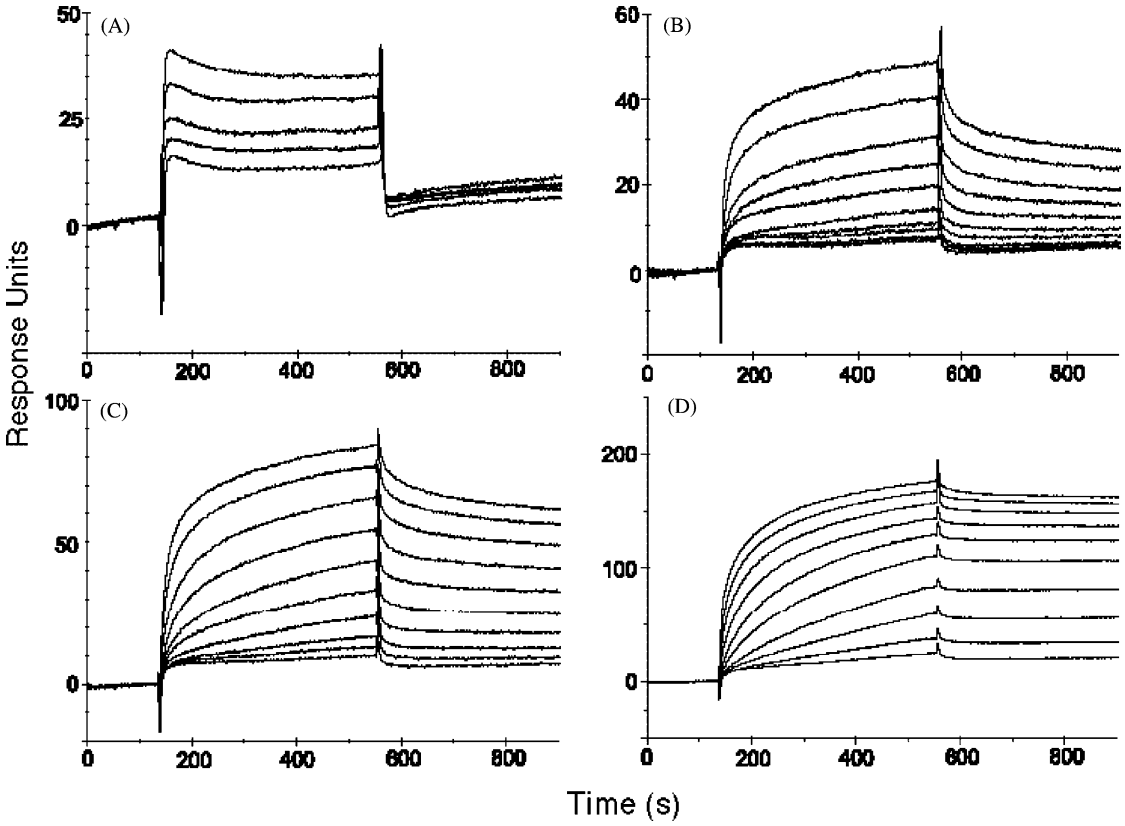

Fig. 1. Surface plasmon resonance analysis of the binding of polysialic acid of different chain lengths to immobilized MenB antibody mAb735. The association, steady-state and dissociation phases are from left to right in each sensorgram (response versus time). The concentrations in each panel are from top to bottom. (A) $Neu5Ac_{16}$ (d.p. 16); 50,000, 25,000, 12,500, 6,250 and 3120 nM. (B) HMW3 (d.p. 30); 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 nM. (C) Colominic acid (d.p. ∼100) and (D) MenB (d.p. ∼200); 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.195 nM.

accordance to the observed the minimum chain length requirement for the antibody (Häyrinen et al., 1989). The maximum responses increased according to chain length. The increase became proportional to mass (RU/d.p. was constant) after d.p. ∼30, suggesting that sufficient affinity to permit saturation at 1 μM polysaccharide concentration was already achieved (see later). In competition experiments, binding of 1 μM colominic acid was also inhibited 20% with simultaneous injection of shorter oligosaccharides (d.p. 5, 7 and 16 at 100 μM; data not shown), although there were no significant differences in the inhibitory effects of various oligomers tested. The results for penta- and hepta-mer are in accordance with the independence of binding affinities on the chain length observed for short oligomers (Evans et al., 1995). A more pronounced inhibition with the longer 16-mer could have been masked by the increase in the observed response due to the binding of the oligomer itself to the antibody (Table 2). This is supported by the slightly higher overall response obtained in the presence of d.p. 16 when compared to d.p. 5 and 7, respectively.

Table 2
Relative maximum binding of oligo- and polysialic acids of different chain length to antibody mAb735[a]

Compound	d.p.	RU	RU/d.p.
$Neu5Ac_{1–10}$	1–10	∼0	n.d.
$Neu5Ac_{11}$	11	0.5	0.04
$Neu5Ac_{12}$	12	0.9	0.08
$Neu5Ac_{13}$	13	1.3	0.10
$Neu5Ac_{14}$	14	2.5	0.18
$Neu5Ac_{15}$	15	3.4	0.23
$Neu5Ac_{16}$	16	4.6	0.29
HMW1	∼31	49.9	1.61
HMW2	∼40	58.6	1.46
HMW3	∼50	95.0	1.90
ColA	∼100	160	1.60
MenB	∼200	∼320	1.60

[a] Determined at standard PSA concentrations of 1 μM.

3.3. Reaction kinetics

For short oligomers (d.p. <16), the rate constants could not be reliably determined due to the narrow analysis windows resulting from their very fast kinetics (Fig. 1, panel A). However, the K_D-values estimated from the approximated rate constants obtained (K_D^{kin}) were similar to the steady-state binding values. The observed fast reaction rates were also in accordance with those previously reported for anti-carbohydrate antibodies (MacKenzie et al., 1996;

Table 3
Binding of polysialic acid to MenB antibody mAb735: Kinetic and steady-state analysis of the binding constants

Compound	d.p.	Kinetic binding[a]			Steady-state binding[b]			
		k_{on} ($\times 10^3\,M^{-1}\,s^{-1}$)	k_{off} ($\times 10^{-3}\,s^{-1}$)	K_D^{kin} ($\times 10^{-9}$ M)	K_D^{mono} ($\times 10^{-9}$ M)	K_D^{bi} ($\times 10^{-9}$ M)	K_D^{avg} ($\times 10^{-9}$ M)	K_D^{tot} ($\times 10^{-9}$ M)
$Neu5Ac_{16}$	16	n.d.[c]	n.d.[c]	8200	n.d.[d]	13000	8260	7400
HMW1	31	36	1.6	46.0	15.0	170.0	92.5	76.0
HMW3	~50	290	1.8	6.2	2.6	47.1	24.9	39.0
ColA	~100	1300	1.6	1.2	2.5	11.0	6.8	2.2
MenB	~200	2000	1.2	0.6	0.4	1.3	0.9	1.3

[a] Calculated by BIA evaluation software after nonlinear curve fitting to the data. $K_D = 1/K_A$.
[b] Calculated by Scatchard analysis using the linear parts of the plots.
[c] Not determined due to narrow analysis window resulting from fast kinetics.
[d] Not determined due to insufficient data points.

Lookene et al., 1996). Longer polymers had progressively lower net association rates calculated for the average size (d.p. 30–200) of the mixtures (Table 3). The values obtained for k_{on} were also in very good agreement with the values calculated from steady-state data ($k_{on} = k_{off}/K_D^{avg}$). In competition experiments, short oligosaccharides inhibited the binding of colominic acid by decreasing the association rate. This can be explained by a fast exchange of the competing oligosaccharides: they slow the association by repeatedly blocking the binding sites. The effect on the dissociation rate is less pronounced, since the long chain polysaccharides already occupy the binding site. Co-injecting short oligosaccharides has actually been used to promote dissociation by preventing the rebinding of larger analytes (MacKenzie et al., 1996).

3.4. Binding affinities

The antibody binding affinity increased rapidly with PSA chain length (Table 3, Fig. 2). For a 16-mer, the $K_D = 8.2 \times 10^{-6}$ M was close to $K_{aff} = 2 \times 10^{-6}$ M described for a similar preparation by solution ligand binding (Häyrinen et al., 1989). The relationship of K_D and d.p. is demonstrated in Fig. 3. The K_D-values interpolated from the kinetic (2×10^{-8} M) and steady-state (5×10^{-8} M) plots corresponding to a 40-mer, are similar and close to the experimentally determined steady-state value of 7.4×10^{-8} M. The binding affinities obtained in the present study were one to two orders of magnitude higher than the K_D-values of 2×10^{-5} and 3×10^{-6} M, calculated for 15- and 41-mer from the previously published association constants (4×10^4

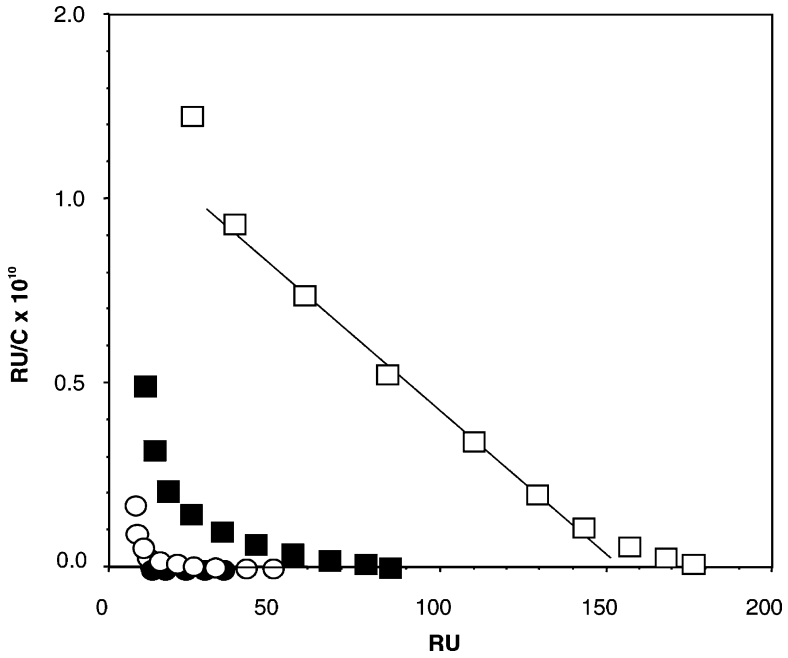

Fig. 2. Modified Scatchard analysis of binding of polysialic acid to the immobilized antibody. $Neu5Ac_{16}$ (●), HMW3, (○), colominic acid (■) and MenB (□). Regression of the linear region ($R^2 = 0.997$) of plot of MenB is indicated by a line. Results are averaged from three individual experiments with duplicate injections of analyte. S.E.M. for determining RU was 3%.

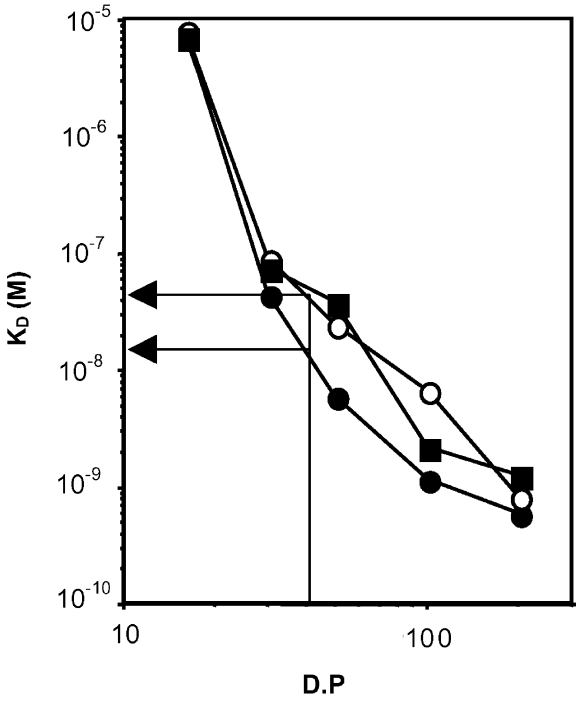

Fig. 3. Relationship of kinetic, average and total antibody binding affinities and polysaccharide chain length. Data are taken from Table 3. K_D^{kin} (●), K_D^{avg} (○) and K_D^{tot} (■), see text for details. Decreasing K_D indicates increasing affinity. Arrows designate the estimation of K_D for a ~40-mer from kinetic and steady-state plots (2×10^{-8} and 5×10^{-8} M, respectively).

and $3 \times 10^5\,M^{-1}$, respectively) (Evans et al., 1995). The difference between the K_D-values for d.p. 16 and 200 was more than 10,000-fold. In previous studies with group B streptococcal (GBS) types II and III capsular polysaccharide fragments, the antibody binding affinity was also shown to increase by polysaccharide chain length, by a factor of ~300, on increasing the d.p. from 2.6 to 92 hexasaccharide repeating units (Michon et al., 1987; Zou et al., 1998). The decrease of saturation binding values with d.p is however different from the results obtained with PSA antibodies.

The biphasic curves obtained in the Scatchard analysis (Fig. 2) indicated concentration-dependent bivalent binding (MacKenzie et al., 1996). This behavior of mAb735 was previously observed with a 41-mer PSA (Evans et al., 1995) and is similar to the binding of heparan sulfate to lipoprotein lipase (Lookene et al., 1996). Although, we have shown previously, that only one antibody combining site is needed for binding of mAb735 to polysialic acids (Häyrinen et al., 1989), both sites of the antibody can be saturated using higher ligand concentrations. Steady-state binding constants were calculated for both lower and higher site occupancy, and the values corresponding to mono- and bivalent binding (K_D^{mono} and K_D^{bi}, respectively) were found to differ by one to two orders of magnitude (Table 3).

Dissociation constants calculated from kinetic data using BIA-evaluation steady-state analysis protocol were similar to those obtained by Scatchard analysis. K_D-values obtained from regression over the whole measuring range using all data points (K_D^{tot}) were very similar to the averages calculated from both phases (K_D^{avg})—a method also suggested previously (Wessels et al., 1987; MacKenzie et al., 1996). Triphasic Scatchard curves, not previously described to our knowledge, were observed for the MenB polysaccharide (Fig. 2). The K_D^{tot} calculated from the linear ($R^2 = 0.997$) region indicates a high average affinity over a wide concentration range.

3.5. Specificity of the interaction

The polysialylated glycopeptides from human embryonal brain material exhibited tight binding of $K_D \sim 5 \times 10^{-9}$ M, suggesting the presence of long chain epitopes in the preparation. The binding was totally abolished by sialidase treatment of the analytes and no binding with control 'normal' (i.e. non-polysialylated) glycopeptides of the same origin was observed, confirming that the binding was specific to PSA-epitopes. The results are in agreement with the demonstrated binding of polysialosyl glycopeptides to PSA-antibodies, including mAb735, in a ligand-binding assay (Finne et al., 1983a,b; Häyrinen et al., 1989). In a previous analysis of a monoclonal anti-PSA IgM antibody (JLP5B9), a similar K_D of 2 nM was obtained for the interaction with the embryonal form of NCAM (Del Rio et al., 1998). The observed unexpectedly high affinity of mAb735 for long PSAs was also reflected in the need for rigorous regeneration after binding of MenB polysaccharide in the SPR, as well as in the difficulties in the removal of bound mAb735 from immunoblots of polysialylated NCAM.[1]

As some gangliosides have been suggested to cross-react with oligosialic acid antibodies (Patel et al., 1989), the reactivity of their oligosaccharide-analogues, mono- and disialosyl lactose was tested. Although high concentrations (up to 10 mM) of disialyllactose, sialyllactose and lactose were used in the assay, no binding could be demonstrated. The results are similar to those obtained earlier in a ligand-binding assay (Häyrinen et al., 1995).

To eliminate the possibility of the observed responses resulting from non-specific electrostatic adherence, we used hyaluronan as a polyanionic ligand analog. No binding was observed even with high concentrations of hyaluronan. This suggests that more than just a polyanionic or polycarboxylic charge-structure is required for the interaction.

3.6. Effects of temperature, pH, ionic strength and buffer

To study the previously reported temperature dependency of antibody binding, the binding assays were performed at various temperatures. No marked differences in maximal responses for colominic acid were observed at 4, 25 or 37 °C, although the signal-to-noise ratio was improved at lower temperatures, probably due to increased gas solubility

[1] Mühlenhoff, unpublished observation.

(less air bubbles) in the buffer. The results are in agreement with our earlier studies (Häyrinen et al., 1989, 1995) and indicate no major temperature dependency of the binding for this antibody. Although, changes in temperature had no apparent effect on the on-rate of reaction, a slightly slower dissociation of the complex was observed at +4 °C. This could account for the reported increase in apparent affinity (Diaz-Romero and Outschoorn, 1993; Mandrell and Zollinger, 1987) as well as avidity (Zollinger, 1998) of MenB antibodies at +4 °C.

The PSA–Ab binding reaction has been suggested to involve electrostatic interaction (Häyrinen et al., 1989; Evans et al., 1995). According to previous studies (Aubin and Prestegard, 1993), the pK_a-values of carboxyl groups in PSA are exceptionally high compared to Neu5Ac-mono- and oligomers: the carboxyl groups are assumed to be 100% protonated at pH 2.5 and 30% at pH 7.0. To study the contribution of charge on the reaction energetics, we varied the composition and pH of the assay buffer, thus changing the protonation state of the carboxylic groups of the PSA. The response for a particular concentration of colominic acid increased by 35% on lowering the pH from 7.9 to 4.5, although no changes were observed in the kinetics or affinities (data not shown). The choice of buffer (HBS, Tris, sodium acetate) did not affect the kinetic or steady-state binding constants. However, slightly higher overall responses were observed in sodium acetate buffer than in HBS. High concentrations of sodium ions (>150 mM NaCl) facilitated the dissociation of the PSA–Ab complex.

3.7. Divalent cations

The recent accumulation of evidence suggests a link between calcium and polysialic acid interactions during neuronal development: Calcium ions have been shown to be involved in the regulation of polysialylation (Brusés and Rutishauser, 1998) and aggregation properties of NCAM (Rouiller et al., 1990; Kiss et al., 1994). Calcium entry is also shown to regulate NCAM isoform expression and polysialylation in the developing muscle (Rafuse and Landmesser, 1996). Interestingly, the polysialic acid moiety of the NCAM was recently shown to be involved in the guidance of retinal axons (Monnier et al., 2001), an event also controlled by Ca^{2+} (Gomez and Spitzer, 1999). As a hygroscopic salt, Ca^{2+} could double the effective hydration volume of PSA—a property that is considered sufficient to explain the role of PSA in NCAM function (Troy, 1992).

The effects of cations were studied by substituting the standard HBS-buffer by acetate or Tris-based buffers and varying the cation concentrations by adding $CaCl_2$, $MgCl_2$, $MnCl_2$ or $ZnCl_2$. We observed a dose-dependent increase in the maximum level of response by Ca^{2+} and Mn^{2+}, whereas Mg^{2+} had less pronounced effects (Fig. 4). Increasing any divalent cation concentration to higher than 25 mM inhibited the binding. This is likely due to increased dissociation of the complex analogous to the effect observed for Na^+.

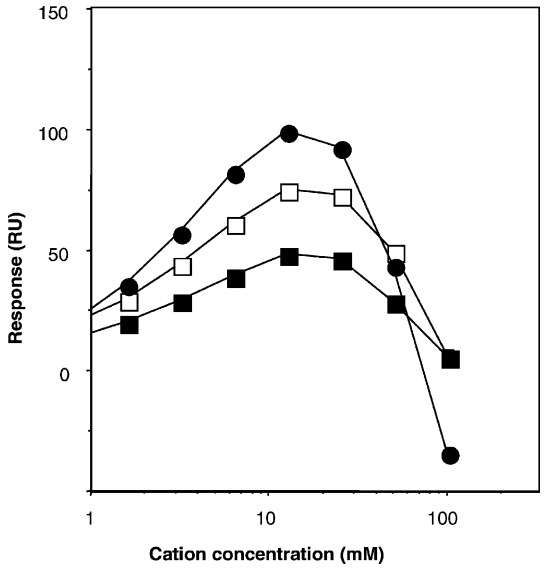

Fig. 4. Effect of divalent cations on the binding of polysialic acid to the immobilized antibody. SPR responses for 1 μM colominic acid were recorded in the presence of varying concentrations of Ca^{2+} (■) and Mg^{2+} (□) or Mn^{2+} (●). Results are averaged from two individual experiments with duplicate injections of analyte. S.E.M. for determining RU was 3%.

No saturation of response with high PSA concentrations due to non-specific adherence to the matrix was observed. However, dose-dependent increases in responses could in theory result from enhancement of PSA self-aggregation.

The effect Ca^{2+} of on relative maximal response was most pronounced at PSA concentrations in the range of 1–100 nM (ratio of Neu5Ac-monomer:divalent cation = 1:10–1000) (Fig. 5). Mg^{2+} induced a response only slightly

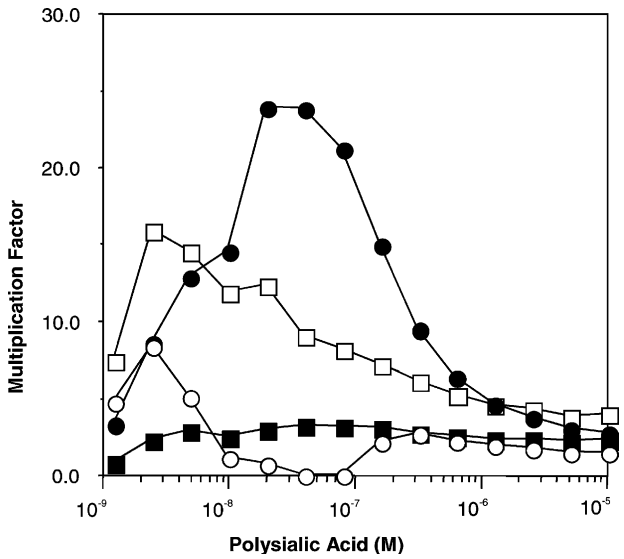

Fig. 5. Effect of polysialic acid concentration and divalent cations on the binding of polysialic acid to immobilized antibody. SPR responses for various concentrations of colominic acid were recorded in the presence of 25 mM Ca^{2+} (■), 10 mM Mg^{2+} (□), 20 mM Mn^{2+} (●) or 20 mM Zn^{2+} (○). Results are averaged from two individual experiments with duplicate injections of analyte. S.E.M. for determining RU was 3%.

Table 4
Effects of divalent cations and spermine on the affinity and relative maximum binding of polysialic acid to antibody mAb735

Compound	Concentration (nM)	Multiplication factor[a]	K_D ion–PSA ($\times 10^{-3}$ M[b])	X^2	K_D PSA–Ab ($\times 10^{-8}$ M[c])
Ca^{2+}	25	4	4.2	0.161	4.4
Mg^{2+}	10	2	8.8	0.011	11.0
Mn^{2+}	20	6	3.7	9.260	8.0
Zn^{2+}	25	6	5.3	4.330	26.0
Spermine	10	10	1.0	0.399	1.4

[a] Multiplication values presented were determined as increased maximum responses (RU) at standard concentration of 1 μM PSA.
[b] Affinity of cation–PSA interaction.
[c] Affinity of PSA–Ab interaction measured in the presence of cation. K_D of control preparation without cations was 1.44×10^{-7} M with $X^2 = 0.047$.

and the effect was not dependent on the concentration of PSA. The effects of Mn^{2+} were stronger, but at slightly higher PSA-concentrations. Ca^{2+} also increased the affinity of PSA–Ab interaction two-fold, whereas Zn^{2+} slightly inhibited the interaction (Table 4). Other cations had no marked effects.

The binding strengths of the cations to PSA were also estimated after subtraction of the non-specific ion–matrix interactions and the contribution of PSA–Ab from the overall dose–response curves (Table 4). Ca^{2+}, Mn^{2+} and Zn^{2+} had comparable affinities of $\sim 4 \times 10^{-3}$ M, whereas affinity for Mg^{2+} was somewhat weaker. We observed two separate binding events for calcium and our estimates for high (0.5×10^{-4} M) and low (4.5×10^{-3} M) affinity constants were in agreement with the previously determined K_D: for Ca^{2+}–colominic acid interaction (10^{-5} and 4×10^{-3} M, respectively) (Shimoda et al., 1994). Since 1 μM colominic acid of d.p. ~100 is suggested to correspond to 10 μM of 'calcium binding sites' of ~10-mer, the maximum effect showing up at 10 μM (i.e. 10^{-5} M) calcium concentration is not unexpected. However, the fact that we could observe the two binding events only in imidazole buffer, but not in Tris, suggests, that the previously observed high-affinity binding—also measured in imidazole-based buffer—(Shimoda et al., 1994) could result artificially from interaction of Ca^{2+} with the imidazole, and not from a second PSA–Ca^{2+} binding event (see later).

We also used NMR to study the possible conformational effects of binding of Ca^{2+} to PSA-oligomers. The signals for NAc–NH, H-3_{axial} and H-$3_{equatorial}$ protons of Neu5Ac-residues were shifted in the presence of Ca^{2+} (Fig. 6). These protons lie below the Neu5Ac pyranose ring plane and are therefore relatively far from the carboxylate groups thought to be responsible for the ionic binding properties of PSA. The results suggest that although Ca^{2+} clearly interacts with PSA, the effects on the conformation are subtle.

3.8. Polyamines and imidazole

The effects of polyamines putrescine (Put), spermidine (Spd) and spermine (Sp) on the PSA–Ab interaction were studied by SPR. The PSA–Ab binding increased parallel to Sp concentration and maximized to 10-fold at 10 mM Sp (Table 4). This is close to the physiological concentration (5 mM) in the brain (Tabor and Tabor, 1984). No further gain in response was observed at higher concentrations. Sp, which is considered the biologically active polyamine, bound with moderate affinity ($K_D \sim 10^{-3}$ M), whereas Put and Spd (precursors of Sp in the polyamine synthesis) had no effect.

Imidazole—a synthetic cyclic imine—had little effect on PSA–Ab even at higher (10 mM) concentrations (data not shown). Additionally, the effects of imidazole could be demonstrated only in the presence of calcium (see earlier). Imidazole was observed to bind non-specifically both to the antibody coated as well as to the activated control surfaces. When 1 μM PSA was co-injected to both antibody-coupled and control surfaces, only the low-affinity component (K_D 4.5×10^{-3} M) of binding of Ca^{2+} to PSA–Ab could be seen after subtraction from the control data. Therefore, we conclude that imidazole had no specific effect on the PSA–Ab per se.

4. Discussion

4.1. PSA–Ab interaction

The high affinities observed for long PSAs could result from their co-operative binding of more than one antibody molecule. The amount of immobilized ligand (11,800 RU or 4.0×10^{14} antibody molecules/m^2) corresponds to average distances of 1000–2000 Å between correctly aligned antibody molecules (assuming random orientation). Thus, co-operative binding could cause the linear region observed in the Scatchard plot of MenB, since an extended 200-mer is generally thought to span about 1200 Å. Actually, this paradigm could reflect the in vivo situation more closely as several adjacent PSA-chains are present both in NCAM N-glycosylation sites and bacterial PSA capsules (Finne et al., 1983a; Jennings et al., 1984). Although, measuring polyvalent affinities is an established method (Mammen et al., 1998), additional information could have been obtained by immobilizing the polysaccharides on the chip and using antibodies and Fab-fragments as analytes. However, this could not be done due to low amount of purified antibodies available. Also, no methods existed at the time for

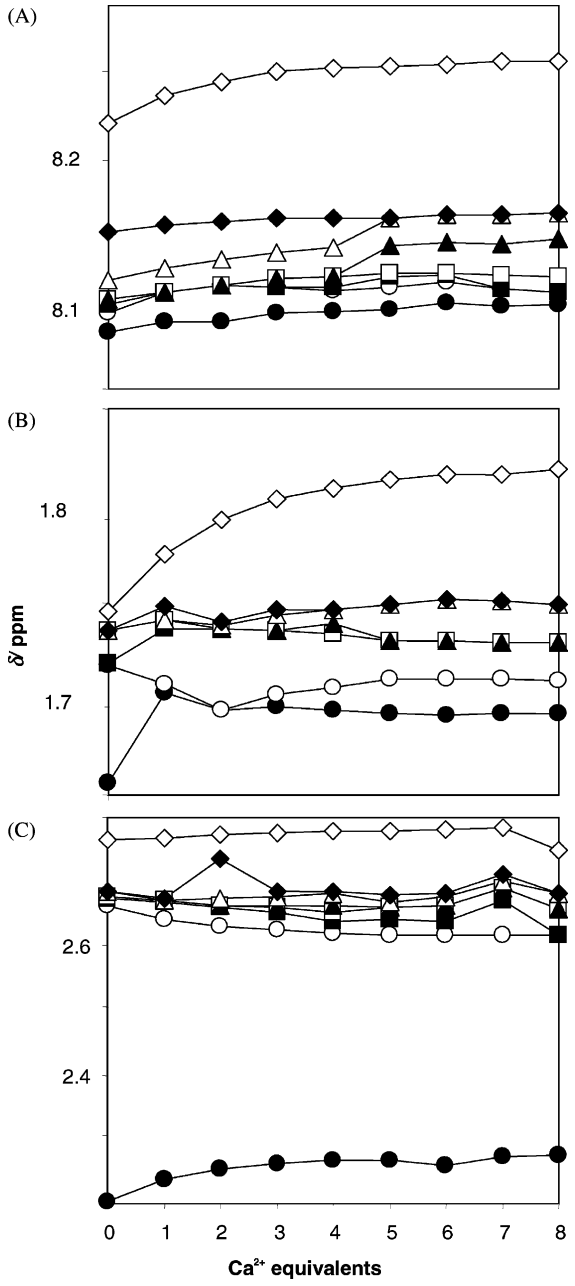

Fig. 6. Calcium-induced changes in the chemical shifts (δ) of some proton NMR signals of $Neu5Ac_8$ oligomers. Chemical shifts of signals from three completely resolved proton species (NAc–NH, H-3_{axial} and H-$3_{equatorial}$) of $Neu5Ac_8$ were determined at 283 K in the presence of Ca^{2+}. Signals originating from the individual eight Neu5Ac-residues are presented with different symbols ((●) to (○)). Signals from different proton species are shown in the corresponding panels: (A) NAc–NH; (B) H-3_{axial}; (C) H-$3_{equatorial}$. The Ca^{2+}-concentration was varied from zero- to eight-fold equivalents that of concentration of the $Neu5Ac_8$ oligomer. Standard error in the determination of δ was <0.01 ppm.

immobilizing and storage of the labile polysaccharides on the chip, without possibly altering the epitope structures present.

Another possible explanation for high affinity of long polysaccharides—in addition to co-operative binding of long polysaccharides to the antibody—is the observed filament bundle formation of PSA-molecules (Toikka et al., 1998): a network of aggregated PSA molecules could bridge adjacent antibody molecules. The aggregate formation of PSA could also contribute to the observed line broadening in ^{1}H-NMR spectra of long-chain PSA. Although Cowman et al. observed similar bundle formation for hyaluronic acid, they suggested this in part to artificially result from enhanced adhesion to mica (used as a matrix in AFM) by low concentrations of divalent cations (Cowman et al., 1998). This is however not the case in SPR, where mica is not employed and non-specific adhesions to matrix are eliminated by subtraction of control data.

Fast association observed for the antibody binding is typical for electrostatic interactions (Evans et al., 1995). The effects of Na^+ and of pH in SPR support the contribution of electrostatics to the overall binding: interactions with a marked electrostatic component are strongly dependent on ionic strength, which affects mainly the dissociation (Lookene et al., 1996). In immunoaffinity chromatography, the PSA glycans of increasing d.p. could be eluted from an immobilized mAb735 column by increasing NaCl concentration (see Section 2). This behavior is similar to their separation in ion-exchange chromatography and was also recently observed for glycosaminoglycans (Manzi et al., 1998). Recently NaCl was shown to inhibit the binding of PSA to the neurotrophic factor BDNF, which supports the involvement of electrostatics in this interaction (Kiss, 1998). Also, Na^+-ions have been shown to counteract the effect of Ca^{2+} in CD spectra of PSA (Shimoda et al., 1994). Our previous observations on the unusually high pI (~9.0) of the mAb735 combining sites further suggest the contribution of electrostatics in the PSA–Ab interaction (Häyrinen et al., 1989). Interestingly, α(2–8)-polysialyltransferase from embryonic chick brain—another PSA-binding molecule—also has a high pI of 10.4–10.5 (Troy, 1992). This is due to a very basic extended region of 31 amino acids (pI 11.6–12.0) thought to participate in the PSA chain extension.

4.2. Calcium and other cations

The previously observed minimal effects of cations on side chain orientation in NMR experiments in magnetically oriented systems (Aubin and Prestegard, 1993) support the view, that Ca^{2+} has no marked effects on the conformation of PSA. However, the authors could not exclude calcium binding, as they had used phosphate buffer, which may prohibit accurate assessment of Ca^{2+}-binding to sialic acid by limiting the amount of free Ca^{2+} (Aubin and Prestegard, 1993, note added in proof). Also, Jaques et al. (1976) note that colominic acid differs from Neu5Ac in respect of calcium binding since the OH-groups involved in glycosidic bond formation can no longer participate in calcium binding. The results from ligand binding and CD-spectroscopy suggest biphasic affinity and reaction sites different from Neu5Ac for the chelating of Ca^{2+} to colominic acid (Shimoda et al.,

1994; Bystricky et al., 1997). Mg^{2+} was also shown not to have any marked effects either to binding or conformation of PSA. Our results do not exclude that Ca^{2+} could stabilize some conformational epitope, possibly a local helix. Since both the antibody and calcium binding sites are suggested to be 10–15-mer, the epitope could be a helical motif with a pitch of 10–15 residues per turn. Ca^{2+} is not required as such for filament bundle formation, since filament bundles are also obtained when carboxylic charges are balanced with Mg^{2+} or Na^{+} ions.[2]

4.3. Polysialic acid and polyamines

Despite decades of research, the physiological role for polyamines is still under debate, although some functions in vivo have already been established (Tabor and Tabor, 1984). Polyamines and PSA are both expressed excessively during the growth and metastasis of brain tumors, and both are charged molecules which are suggested to involve helical motifs in their interactions: PSA adopts a helical conformation in solution and Sp is thought to compact and stabilize the DNA double helix by bridging negative charges of the sugar phosphate backbone (Evans et al., 1995; Mantione et al., 1990). Interestingly, spermine has been used routinely as a co-crystallizing agent in X-ray diffraction of sialylated glycoproteins, and recently as matrix in MALDI–MS-analysis of acidic glycans containing sialic acids (Mechref and Novotny, 1998). Our results may suggest a new role for polyamines in the developing nervous system as ‘counter-ions’ of PSA. The interaction of polyamines with PSA could be involved for example in the neuro-protective effects of ornithine decarboxylase activation in neuronal plasticity and re-nervation after ischemia (Lukkarinen et al., 1999).

5. Conclusions

Our results are in agreement with the suggested selection of long epitopes by immune system in the production of protective antibodies against MenB (Evans et al., 1995). A similar requirement for long chains was observed also with antibodies generated against the N-propionylated derivative of MenB polysaccharide (Granoff et al., 1998; Bruge et al., 1998). The antibodies were bactericidal in vitro and could readily enter the fetal circulation through the placenta. Although the actual hazards of the cross-reactivity in vaccination remain to be evaluated, our results emphasize the importance of elucidating the nature and regulation of PSA-epitopes during human brain development. Studies to determine the structures and conformations of these glycans are in progress.

Our data suggests that the observed better avidity of the protective anti-MenB antibodies result from their higher affinity to long PSA molecules. Whether the high affinity is due to mono- or polyvalent binding is irrelevant to the biological effects, as the net avidity is a result of all interactions. The effects of cations are intriguing and imply, that although calcium is not likely needed to induce a conformational epitope in PSA, it could stabilize one. Rather the long conformational epitope could facilitate the binding of calcium, which in turn would promote the observed physiological functions of PSA. The results presented here support a synergistic role of NCAM–PSA, polyamines and Ca^{2+} in the developing nervous system.

[2] Toikka and Finne, unpublished observation.

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