Characterization of O-Glycosidically Linked Oligosaccharides of Rat Erythrocyte Membrane Sialoglycoproteins†

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Received April 21, 1986; Revised Manuscript Received August 12, 1986

ABSTRACT: The carbohydrate units of the rat erythrocyte membrane sialoglycoprotein rSGP-4 [Edge, A. S. B., & Weber, P. (1981) *Arch. Biochem. Biophys. 209*, 697–705] have been characterized. All of the carbohydrate of this M_r 19 000 glycoprotein occurs in O-glycosidic linkage to the peptide; following alkaline borohydride treatment and chromatography on Bio-Gel P-2, sialic acid containing oligosaccharides terminating in *N*-acetylgalactosaminitol were obtained. Their structures were determined by compositional analysis, exoglycosidase digestions, alkaline sulfite degradation, and periodate oxidation. The oligosaccharides were characterized for molecular weight and linkage by direct chemical ionization and gas–liquid chromatography/mass spectrometry, respectively. The structures are proposed to be NeuAcα2→3Galβ1→3GalNAc-ol, Galβ1→3(NeuAcα2→6)GalNAc-ol, NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAc-ol, and NeuAcα2→3Galβ1→3(NeuAcα2→3Galβ1→4GlcNAcβ1→6)GalNAc-ol. Two of the *N*-acetylglucosamine-containing hexasaccharides were present per molecule of rSGP-4 along with two trisaccharides and seven tetrasaccharides.

In a previous study the major sialoglycoproteins of the rat erythrocyte membrane were isolated and characterized (Edge & Weber, 1981). The smallest of these glycoproteins, designated rSGP-4[1] (M_r 19 000), was obtained in homogeneous form and was found to be 68% carbohydrate with the oligosaccharide units attached in O-glycosidic linkage to serine and threonine. A distinctive feature of the rat erythrocyte membrane sialoglycoproteins was the occurrence of O-acetylated *N*-acetylneuraminic acids.

Erythrocyte membrane sialic acid has been shown to be a modulator of erythropoiesis (Skutelsky & Farquhar, 1976) and erythrocyte clearance (Gregoriadis et al., 1974; Aminoff et al., 1977) in the rat. The erythrocyte membrane sialoglycoproteins are major carriers of cell surface sialic acid (Skutelsky & Farquhar, 1976; Edge & Weber, 1981), and it is likely that the sialic acid containing oligosaccharide chains of the glycoproteins on the red cell surface are involved in the recognition processes that underlie these cellular interactions.

Relatively few detailed studies on the carbohydrate portion of isolated, well-defined cell surface glycoproteins have been performed. This is in contrast to the extensive characterization of the more easily obtained secreted glycoproteins, including both the plasma glycoproteins containing primarily N-glycosidically linked carbohydrate chains and the mucins that contain O-glycosidically linked carbohydrates. Because of the widespread use of rat erythrocytes in biochemical and physiological studies, it would be of interest to determine the structures of the oligosaccharide chains of the purified rat erythrocyte membrane glycoproteins in order to correlate this structural information with the proposed functions of these glycoconjugates. This paper describes the structural determination of the major O-glycosidically linked oligosaccharides present in these glycoproteins.

Materials and Methods

Materials. Chondrosine, 2-aminopropanediol, ribulose *o*-nitrophenylhydrazone, methylene chloride, triethylsilane, ethereal boron trifluoride, neuraminyllactose, Purpald, *Clostridium perfringens* neuraminidase, jack bean β-galactosidase, and jack bean β-*N*-acetylglucosaminidase were obtained from Sigma Chemical Co. Sodium cyanoborohydride was from Aldrich Chemical Co. Sulfonylhexosamine was prepared as described previously (Weber & Winzler, 1970). Reduced, β-eliminated oligosaccharide standards were prepared from tryptic peptides derived from human MM-active erythrocytes (Winzler et al., 1967; Thomas & Winzler, 1969), and a column of Bio-Gel P-2 (−400 mesh) (Bio-Rad) was used for their separation.

The rat erythrocyte sialoglycoproteions were isolated as previously described (Edge & Weber, 1981). The sialoglycoprotein fraction prepared by phenol–water partitioning and ion-exchange chromatography on SP-Sephadex has been designated rSGP and the purified M_r 19 000 glycoprotein obtained by gel filtration on Sephadex G-50 rSGP-4. This glycoprotein contains *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, fucose, *N*-acetylneuraminic acid, and *N*,*O*-acetylneuraminic acids in a ratio of 1.0:0.2:1.2:0.1:1.9:0.6 (Edge & Weber, 1981).

β-Elimination Reaction. The glycoproteins were dissolved in 0.1 N NaOH/1.0 M sodium borohydride (Carlson, 1968) and incubated at 45 °C for time periods ranging from 18 to 72 h. To monitor the extent of reaction, aliquots were taken

†Supported in part by NIH Grants AM 27767 and BRSG SO7RRO5394.
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[1] Abbreviations: NeuAc or NANA, *N*-acetylneuraminic acid; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-galactose; Glc-ol, D-glucitol; GalNAc-ol, *N*-acetyl-D-galactosaminitol; rSGP, rat sialoglycoprotein; FID, free induction decay.

0006-2960/86/0425-8017$01.50/0

and hydrolyzed in 4 N HCl as described below for hexosamines and hexosaminitols.

After the time course of the reaction was established, reduced oligosaccharides were prepared by alkaline borohydride treatment for 24 h. At this time the reaction mixture was cooled in an ice bath and neutralized with dilute acetic acid after a drop of 1-octanol had been added to prevent foaming. The mixture of reduced oligosaccharides and peptides was passed through a column of Dowex 50-X2 (H^+) (2 mL of resin/mL of reaction mixture) in the cold. The column was eluted with 3 bed volumes each of water and 50% methanol to elute the oligosaccharide fraction, followed by 1 M NH_4OH to elute peptidic material. After lyophilization of the eluted fractions, the samples were redissolved in methanol and evaporated several times to remove boric acid as its methyl ester.

Gel Filtration on Bio-Gel P-2. The sample was applied to a 2 × 100 cm column of Bio-Gel P-2 (−400 mesh), and the column was pumped at 21 mL/h with 0.1 M pyridine–acetate, pH 5.5. The stream was diverted at 3 mL/h via a stream-splitting "T" to a detection system consisting of a Technicon multichannel autoanalyzer pump with a manifold for periodate oxidation and Purpald reaction (Weber, 1979) while the remaining sample was collected in 10-min fractions.

In certain experiments a portion of the effluent stream (3 mL/h) was also diverted via a stream splitter for alkaline hydrolysis by mixing it with 1 N NaOH (25 mL/h) and heating it in a reaction coil at 100 °C for 40 min. Amine-containing components were detected in a fluorometer after being mixed with the *o*-phthalaldehyde reagent (Benson & Hare, 1975) prepared in 0.8 M boric acid (36 mL/h).

Alkaline Sulfite Treatment. β-Elimination in the presence of sodium sulfite was carried out by dissolving 0.5 mg of glycoprotein in 0.2 mL of 0.5 M sodium sulfite in 0.1 N NaOH and incubating the mixture at room temperature in the dark for 72 h. The reaction mixture was brought to ice-bath temperature, the pH was adjusted to 5 by the addition of 2.0 M pyridine–acetate buffer, pH 3.5, and cationic compounds were removed by passage through Dowex 50-X2 (H^+) (2 mL of resin/mL of reaction mixture) at 4 °C. The column was washed with 50% methanol and the eluate immediately adjusted to pH 5 with pyridine.

Periodate Oxidation of Reduced Oligosaccharides. Samples of oligosaccharides (15–30 nmol) were dissolved in 0.5 mL of 50 mM sodium metaperiodate in 50 mM sodium acetate buffer, pH 4.5. The oxidation was carried out at room temperature in the dark for 18 h. To terminate the reaction, glycerol was added; the pH was then raised to 7–8 by addition of a 0.1 M sodium borate buffer (pH 9.5), and a 30-fold molar excess of sodium borohydride over the periodate was added. The reduction was allowed to proceed for 18 h at which time the borohydride was destroyed by the addition of glacial acetic acid. The sample was then passed through a mixed-bed ion-exchange column containing 1 mL of Dowex 1-X10 (formate) and 0.5 mL of Dowex 50-X8 (H^+). The eluate was evaporated several times with methanol and hydrolyzed for sugar analyses. A threosaminitol standard was prepared by acetylation (pyridine–acetic anhydride, 1:1 v/v) and reduction with sodium borohydride of chondrosine. Following periodate oxidation the products were reduced and hydrolyzed. A mixture of arabinosaminitol and ribosaminitol was prepared by reductive cleavage of ribulose *o*-nitrophenylhydrazone (4 mg) in 5 mL of 10% ethanol containing 2 mM HCl, 8 mM palladium chloride, and 0.2 M sodium borohydride. The product eluted as a single peak from the cation-exchange column used for separation of the amino sugar alcohols, allowing identification of the five carbon species.

Exoglycosidase Digestions. Digestions of oligosaccharides (25 nmol) with exoglycosidases were done at 37 °C for 24 h in a volume of 100 μL of 0.1 M sodium acetate buffer, pH 5.0. The quantities of enzyme used were as follows: neuraminidase, 20 milliunits; β-galactosidase, 200 milliunits; β-*N*-acetylglucosaminidase, 200 milliunits. In some cases mild acid hydrolysis (0.1 N H_2SO_4, 80 °C, 1 h) was used for sialic acid removal. The digested oligosaccharides were recovered by passage through Dowex 1-X2 (formate) after mild acid or neuraminidase treatment or by filtration through a 1 × 30 cm column of Bio-Gel P-2 after treatment with the other enzymes.

Permethylation, Reductive Cleavage, and Acetylation. The methylation of the sample was performed by the general procedure of Hakomori (1964) as modified by Sanford and Conrad (1966). The potassium salt form of the anion was prepared (Finne et al., 1980), and methylations were carried out twice for 30-min intervals. The reaction was cooled to 0 °C before addition of the methyl iodide. Excess methyl iodide was removed under a stream of nitrogen prior to the second methylation step. For the reductive cleavage, 100 μL of a homogeneous mixture of methylene chloride (dried over CaH_2), triethylsilane, boron trifluoride etherate, and trifluoroacetic acid (10:11:9:2) was added to a dried residue of the permethylated oligosaccharide according to the previously outlined procedure (Van Langenhove & Reinhold, 1985). Acetic anhydride was used for the acetylation.

Gas Chromatography and Mass Spectrometry. Gas chromatography was carried out on a 30-m fused silica capillary column with DB-225 and DB-1 liquid phases (J. W. Scientific, Rancho Cordova, CA). The gas chromatographic conditions were as follows: for the DB-1 column, initial temperature 75 °C with a 2-min hold and then programmed to 300 °C, at a rate of 10 deg/min; for the DB-225 column, initial temperature 75 °C with a 2-min hold and then rapidly brought to 200 °C at a rate of 29 deg/min. After a 5-min hold, the peaks of interest were eluted with a temperature program of 3 deg/min to the final temperature of 240 °C. Retention times were determined relative to the *n*-alkane $C_{16}H_{34}$.

The mass spectrometer used in this study was a Finnigan-MAT 312 (San Jose, CA) double-focusing instrument fitted with a combined electron and chemical ionization source. For ammonia direct chemical ionization analyses, a programmable power supply was used for ramping the wire current. To maximize the desorption of intact neutral molecules, this wire was coated with a polyimide surface (Reinhold & Carr, 1982). Solutions containing the sample material were added to the coated wire surface, air-dried, and inserted directly into the chemical ionization chamber of the ionization source. For gas–liquid chromatography/mass spectrometry, the columns were directly coupled to the mass spectrometer, and eluting peaks were ionized by either chemical or electron ionization. The reagent gas used while in the chemical ionization mode was ammonia with an ion source temperature of 125 °C. Electron ionization voltage was 70 eV, and the ion source temperature was 200 °C.

Analytical Procedures. Neutral sugars were reductively aminated to glycamines with ammonium sulfate and sodium cyanoborohydride after acid hydrolysis and were analyzed by cation-exchange chromatography with borate buffers (Hara et al., 1979) and an *o*-phthalaldehyde system for detection (Benson & Hare, 1975; Edge et al., 1981). Hexosamines were quantified fluorometrically after acid hydrolysis and cation-exchange chromatography as previously described (Weber &

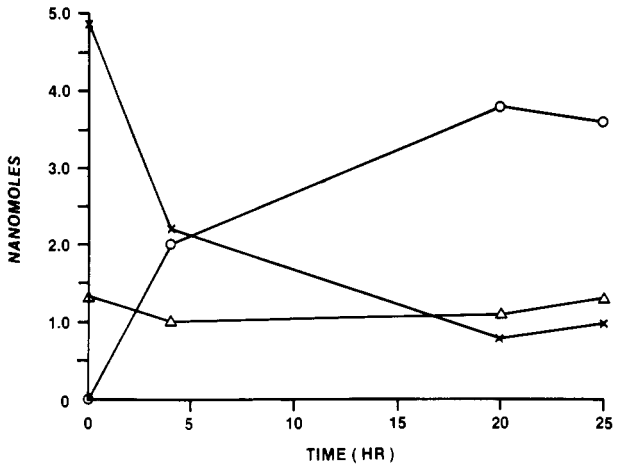

FIGURE 1: Time course of β-elimination of O-linked oligosaccharides from rSGP-4. The glycoprotein (0.5 mg) was incubated in 1 M $NaBH_4$ and 0.1 N NaOH at 45 °C. Aliquots (20 μL) of the reaction mixture were hydrolyzed and analyzed at 0, 4, 20, and 25 h for galactosaminitol (○), galactosamine (×), and glucosamine (△).

Winzler, 1969; Edge & Weber, 1981). *N*-Acetylneuraminic acid was analyzed by anion-exchange chromatography with reducing sugar detection (Sinner & Puls, 1978) and by the thiobarbituric acid assay (Aminoff, 1961) after hydrolysis of the oligosaccharides with 0.05 N H_2SO_4 for 1 h at 80 °C. The sulfonated hexosamine derivative was determined on the amino acid analyzer after hydrolysis with 6 N HCl at 110 °C for 22 h (Weber & Winzler, 1970).

The column used for the separation of the amino sugar alcohols had a 0.9 × 16 cm resin bed of Durrum DC 6A resin (Dionex Corp.) and was eluted with sodium citrate buffer, pH 4.5 (0.35 M sodium concentration), at 60 mL/h and 65 °C. This system was similar to that described by Lee and Scocca (1972). Detection of amino sugars in the effluent was with the *o*-phthalaldehyde reagent.

Results

Isolation of Oligosaccharides Released by Alkaline Borohydride Treatment of rSGP-4. The time course of β-elimination of rSGP-4 O-linked saccharides was followed by analysis for amino sugars at various intervals after the addition of NaOH–$NaBH_4$. Within 20 h, 85% of the galactosamine was lost (Figure 1) and was quantitatively recovered as galactosaminitol. The unreduced galactosamine remained attached to the peptide moiety that was eluted from Dowex 50 after recovery of the released oligosaccharides from the column. The glucosamine value was unchanged during alkaline borohydride treatment; moreover, 80% of this amino sugar was contained in the released oligosaccharide fraction, suggesting that the glucosamine of rSGP-4 was a component of the O-linked carbohydrate units as proposed before (Edge & Weber, 1981).

Table I: Composition of O-Linked Oligosaccharides Released from rSGP-4 by Alkaline Borohydride Treatment[a]

monosaccharide	mol/mol of oligosaccharide		
	S-I	S-II	S-III
galactosaminitol[b]	1.0	1.0	1.0
glucosamine	1.1	0	0
galactose	1.8	1.0	0.9
sialic acid	1.6	1.6	0.9

[a] The oligosaccharides were isolated by chromatography on Bio-Gel P-2. [b] Compositions are reported relative to a galactosaminitol value of 1.0

Filtration of the oligosaccharide fraction from rSGP-4 on Bio-Gel P-2 resulted in the separation of three major peaks designated S-I, S-II, and S-III (Figure 2a), which were detected by automated monitoring for formaldehyde with the Purpald reagent following periodate oxidation (Weber, 1979). These oligosaccharides had respective M_r of 1280, 970, and 670 as estimated by their elution position from the calibrated column; peaks S-II and S-III cochromatographed with the tetrasaccharide and trisaccharide from human glycophorin, NeuAcα2→3Galβ1→3(±NeuAcα2→6)GalNAc-ol (Thomas & Winzler, 1969). Three peaks eluting at similar positions were obtained when rSGP as opposed to rSGP-4 was subjected to the same treatment (Figure 2b). In this case an excluded peak (4.5 h) was also apparent and consisted of acidic glycopeptides containing N-linked chains that are present in the erythrocyte glycoproteins other than rSGP-4 (Edge & Weber, 1981); this peak was shown to contain amino acids following acid hydrolysis.

Composition of O-Linked Oligosaccharides. The oligosaccharides isolated from rSGP-4 by gel filtration contained galactosaminitol, glucosamine, galactose, and sialic acid (Table I). No galactosamine was present, indicating that this amino sugar occurred only at the reducing end of the carbohydrate units. Both S-II and S-III lacked glucosamine and contained

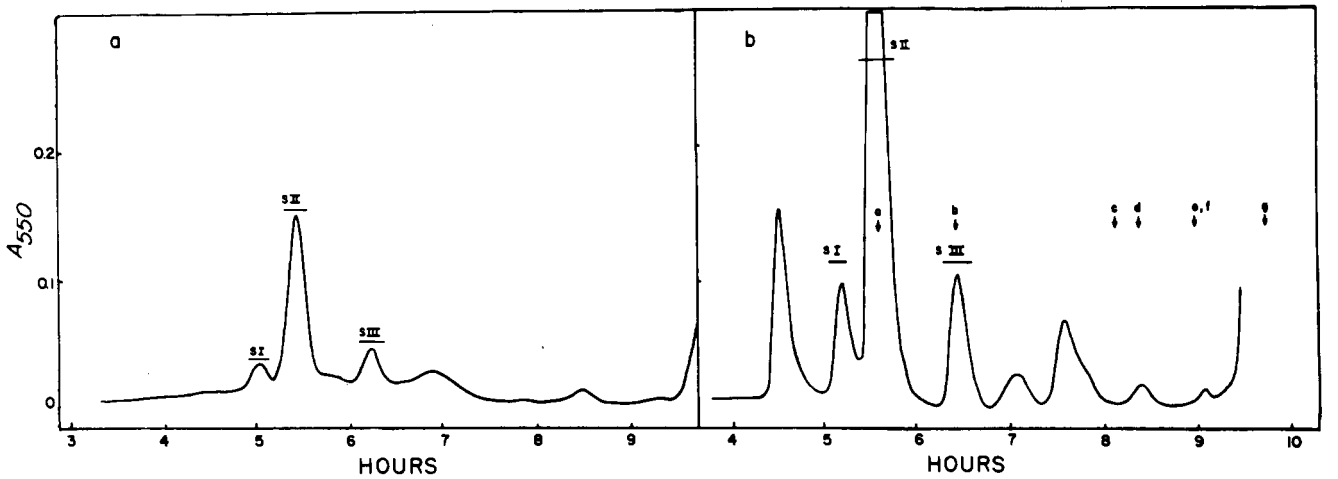

FIGURE 2: Chromatography of reduced oligosaccharides on Bio-Gel P-2. The saccharide fractions from rSGP-4 (a) and from total rSGP (b) were applied to a 2 × 100 cm column and eluted at a flow rate of 21 mL/h with 0.1 M pyridine–acetate, pH 5.5. The components were detected with the periodate/Purpald system described under Materials and Methods. Standards used for calibration of the column (arrows in panel b) include NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAc-ol (a), NeuAcα2→3Galβ1→3GalNAc-ol (b), Galβ1→3GalNAc-ol (c), NeuAc (d), GalNAc-ol (e), Galβ1→4Glc-ol (f), and Glc-ol (g). The excluded volume of the column is at 4.5 h.

Table II: Composition of Oligosaccharides Produced by Exoglycosidase Digestions of Oligosaccharide S-I[a]

	treatment			
monosaccharide	neuraminidase[b]	neuraminidase + β-galactosidase[c]	neuraminidase + β-*N*-acetylglucosaminidase	neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase
galactosaminitol	1.0	1.0	1.0	1.0
glucosamine	1.1	1.2	1.2	0.3
galactose	1.8	0.6	1.7	0.7

[a] Values are expressed as mol/mol of S-I. [b] Treatment with neuraminidase was followed by passage through Dowex 1-X2 to obtain the neutral oligosaccharide. [c] The product of neuraminidase treatment was digested with the indicated exoglycosidases, and the saccharides were reisolated by chromatography on Bio-Gel P-2.

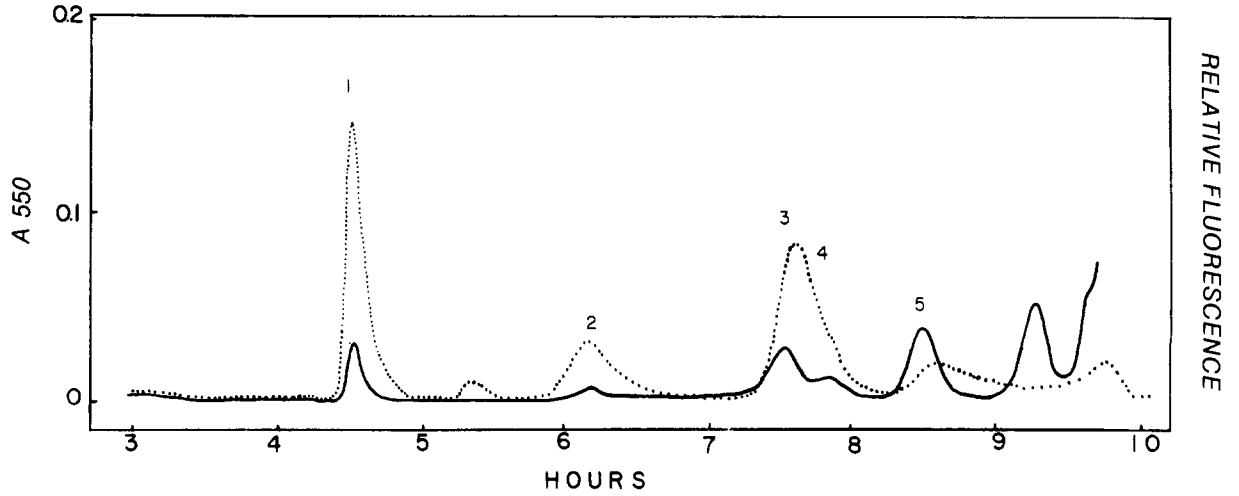

FIGURE 3: Products of alkaline sulfite degradation of rSGP-4 chromatographed on a column of Bio-Gel P-2. Reaction with alkaline sulfite was carried out for 72 h as described under Materials and Methods, and conditions for chromatography were as in Figure 2. The solid line was obtained by monitoring with the periodate/Purpald system (A_{550}) and the dotted tracing with alkaline hydrolysis followed by reaction with *o*-phthalaldehyde (relative fluorescence).

a 1:1 ratio of galactose and galactosaminitol with either one (S-III) or two (S-II) sialic acid residues attached. Oligosaccharide S-I also had two residues of sialic acid and, in addition, contained the glucosamine noted in the released saccharide fraction.

Stepwise Removal of Sugars by Mild Acid and Exoglycosidase Treatment. Both S-II and S-III were converted by mild acid treatment (0.1 N H_2SO_4, 80 °C, 1 h) to a disaccharide that eluted from Bio-Gel P-2 at the position of standard Galβ1→3GalNAc-ol (data not shown); equimolar galactose and galactosaminitol were obtained upon strong acid hydrolysis of this peak. Mild acid or *C. perfringens* neuraminidase treatment of S-I yielded a tetrasaccharide with the composition $(Gal)_2(GlcNGalN\text{-}ol)_1$ (Table II). Digestion of this tetrasaccharide with jack bean β-galactosidase removed one galactose residue completely and the second galactose partially. Jack bean β-*N*-acetylglucosaminidase was only effective in removing *N*-acetylglucosamine from neuraminidase-treated S-I after β-galactosidase treatment (Table II); the second galactose remained bound to GalNAc-ol even under these conditions, indicating that the arrangement of the sugars in the tetrasaccharide was Gal→GlcNAc→(Gal→GalNAc-ol). A galactose residue linked β1→3 to *N*-acetylgalactosaminitol has been shown to be resistant to β-galactosidase digestion in other studies (Van den Eijnden et al., 1979), and indeed, the jack bean β-galactosidase employed in this study manifests only limited activity toward Galβ1→3GlcNAc sequences (Li et al., 1975). Thus the sensitivity of the S-I Gal→GlcNAc linkage to the enzyme suggests that it represents a β-1,4 or β-1,6 isomer.

Linkage Analysis of Oligosaccharides by Alkaline Sulfite Treatment and Periodate Oxidation. Following alkaline sulfite treatment of rSGP-4 and filtration on Bio-Gel P-2, the profile shown in Figure 3 was obtained by periodate/Purpald as well as amine-specific detection. Peak 1 consisted of amino acids after hydrolysis and represented the sulfonated peptide moiety. The only glucosamine-containing fraction was peak 2; it was thus derived from S-I, and its composition indicated that it was a tetrasaccharide composed of sialic acid, galactose, glucosamine, and sulfonylhexosamine. On the basis of the earlier reported mechanism of alkaline sulfite degradation of O-linked saccahride units, a branch attached through C-6 of a 3,6-disubstituted *N*-acetylgalactosamine is released as a fragment terminating in sulfonylhexosamine (Edge & Weber, 1984). In conjunction with the exoglycosidase data, the branching pattern of S-I is therefore NeuAc→Gal→GlcNAc→6(NeuAc→Gal→3)GalNAc-ol.

Peaks 3–5 in Figure 3 consisted respectively of a disaccharide of sialic acid and sulfonylhexosamine, a disaccharide containing sialic acid and galactose, and free sialic acid. The neuraminylsulfonylhexosamine (peak 3) was thought to be derived from S-II and S-III, which would thus contain a NeuAcα2→6(Galβ1→3)GalNAc-ol sequence (Edge & Weber, 1984).

The presence of a substituent at C-3 of *N*-acetylgalactosaminitol in S-II and S-III was confirmed by periodate oxidation of asialo-S-II. Following acid hydrolysis, the amino sugar was recovered as threosaminitol (Figure 4). Furthermore, the galactose residue in intact S-II was not oxidized, indicating that sialic acid occupied C-3 of this sugar, whereas 40% of the galactose in S-III was oxidized, suggesting that it consisted of two isomers with sialic acid occurring either at C-3 of galactose or at C-6 of *N*-acetylgalactosaminitol.

Periodate oxidation of sialic acid free S-I did not affect the *N*-acetylglucosamine residue whereas 95% of this amino sugar was destroyed by periodate treatment of the β-galactosi-

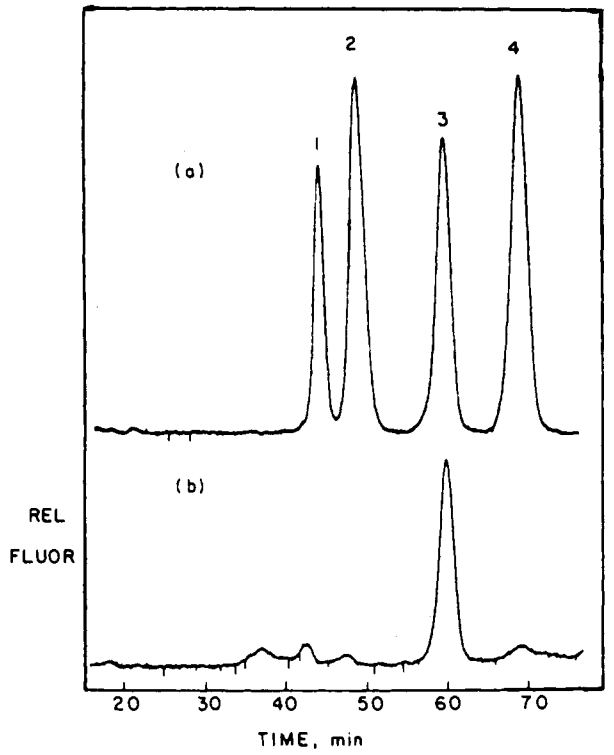

FIGURE 4: Ion-exchange chromatography of products resulting from periodate oxidation of galactosaminitol. (a) Standard amino sugar alcohols were chromatographed: galactosaminitol (1), arabinosaminitol (2), threosaminitol (3), and 2-aminopropanediol (4). (b) Product of periodate oxidation, borohydride reduction, and asialo S-II hydrolysis was applied to the column. Chromatography was carried out as described under Materials and Methods, and detection was with the *o*-phthalaldehyde reagent (relative fluorescence).

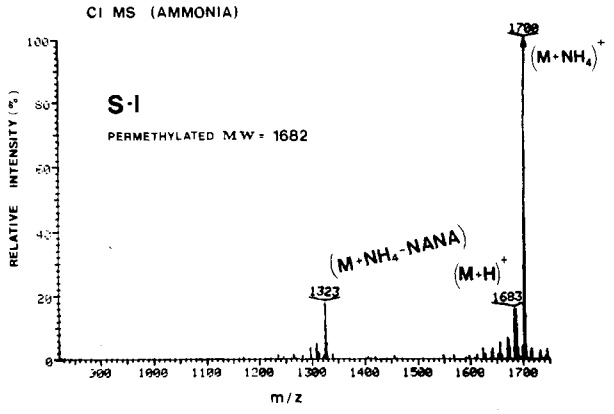

FIGURE 5: Ammonia direct chemical ionization mass spectra (CI/MS) of permethylated S-I. *N*-acetylneuraminic acid is denoted as NANA.

dase-digested sialic acid free saccharide. This result rules out the occurrence of a 6-substituted *N*-acetylglucosamine and in conjunction with the exoglycosidase data allows the assignment of a Galβ1→4GlcNAc linkage.

Mass Spectral Analysis of Oligosaccharides S-I and S-II. The ammonia direct chemical ionization of permethylated S-I is shown in Figure 5. The abundant ions at m/z 1683 [$(M + H)^+$] and 1700 [$(M + NH_4)^+$] indicate the molecular weight of the permethylated derivative to be 1682. This molecular weight is consistent with a hexasaccharide of composition $(NeuAc)_2(hexose)_2(hexose\text{-}NAc)_1(hexose\text{-}NAc\text{-}ol)_1$. The ion at m/z 1323 can be accounted for as loss of a terminal neuraminyl residue ($[(M + NH_4) - NANA]^+$). The mass spectrum of permethylated S-II showed major ions at m/z 1234 [$(M + H)^+$] and 1251 [$(M + NH_4)^+$], which indicated the molecular weight of this material to be 1233, consistent

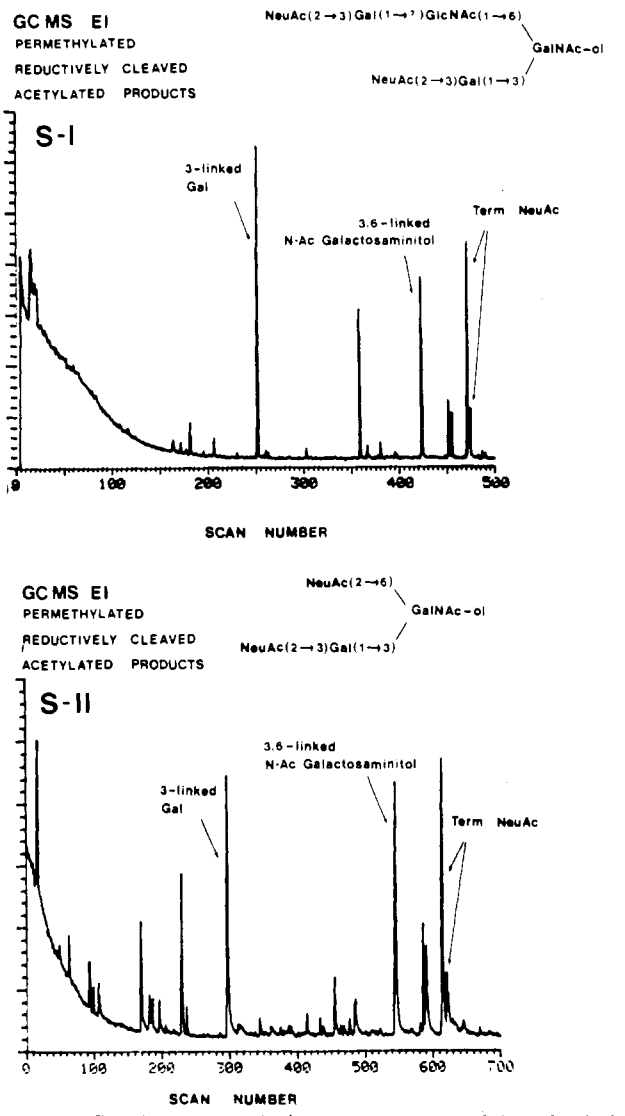

FIGURE 6: Gas chromatography/mass spectrometry of the reductively cleaved, acetylated products of permethylated S-I and S-II. Gas chromatographic conditions were as described under Materials and Methods. A DB-1 capillary column was used.

with a tetrasaccharide that contains one less hexose and amino sugar residue than S-I.

Linkage Analysis by Reductive Cleavage of Permethylated S-I and S-II. Gas chromatographic analysis of the reductively cleaved, acetylated products of permethylated S-I and S-II on a DB-225 capillary column provided the linkage information for the galactose residues. Comparison of gas chromatographic retention times with previously published results (Van Langenhove & Reinhold, 1985) confirmed the presence of a 3-linked galactose with the detection of 1,5-anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol, the reductively cleaved product. A DB-1 capillary column allowed operation at higher temperatures and was found more suitable for the analysis of the higher molecular weight and less volatile neuraminic acid residues; the data thus complement our previously published study of neutral sugars on a DB-225 column.

The total ionization traces obtained by gas chromatography mass spectrometry (DB-1 column) of the reductively cleaved, acetylated products of permethylated S-I and S-II are shown in Figure 6. The presence of terminal *N*-acetylneuraminic acid in both oligosaccharides was suggested by the characteristic doublet that is typical for keto sugars (Van Langenhove & Reinhold, 1985; Rolf & Gray, 1984). The retention times (absolute, 21.18/21.31 min; relative to $C_{16}H_{34}$, 1.50/1.51) and

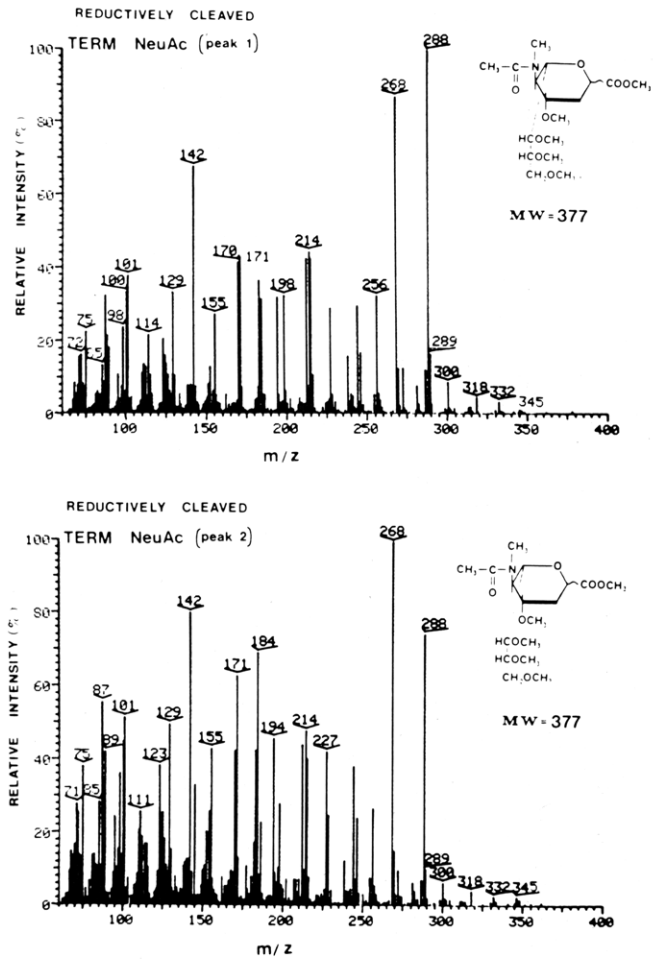

FIGURE 7: Electron ionization mass spectra of the reductively cleaved terminal *N*-acetylneuraminic acid residues.

mass spectra proved to be identical with those obtained by reductive cleavage of permethylated *N*-acetylneuraminic acid and of a model compound, neuraminyllactose. The mass spectral analysis of each peak supported the fact that these products were reductively cleaved, terminal *N*-acetylneuraminic acid isomers. The chemical ionization data indicated a molecular weight of 377 (data not shown), and the electron ionization mass spectra (S-I, scan numbers 471 and 474; S-II, scan numbers 614 and 622) were consistent with the structure (Figure 7).

The reductively cleaved product of the reducing-end galactosaminitol residue 1,4,5-tri-*O*-methyl-3,6-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol eluted with an absolute retention time of 16.99 min (1.20 relative to $C_{16}H_{34}$). The electron ionization fragmentation pattern (S-I, scan number 424; S-II, scan number 545) supports the structure and position of the acetyl groups (Figure 8) (Schwarzmann et al., 1973).

Using the above described methodology, we could not determine the linkage of the *N*-acetylglucosamine residue in S-I. The gas chromatography/mass spectrometry profiles of the reductively cleaved products of S-I failed to indicate an acetylated anhydroalditol derived from this amino sugar. Previous experience has indicated that reductive cleavage of amino sugar residues follows different kinetics than those observed for neutral sugars (Van Langenhove & Reinhold, 1985).

In addition to the aforementioned expected products, an acyclic compound with a molecular weight of 350 was observed (ammonia chemical ionization data, abundant ions at *m/z* 351 and 368) with an absolute retention time of 17.00 min (relative to $C_{16}H_{34}$, 1.21). The product was identified as 1,3,5-tri-*O*-

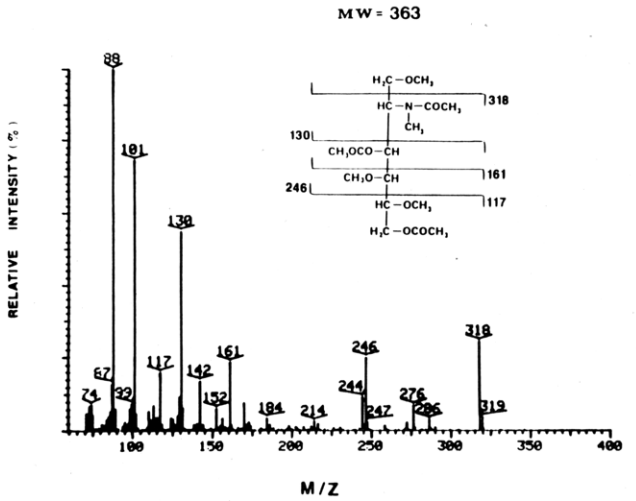

FIGURE 8: Electron ionization mass spectrum of 1,4,5-tri-*O*-methyl-3,6-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol.

acetyl-2,4,6-tri-*O*-methyl-D-glucitol from its electron ionization mass spectrum (S-I, scan number 359; S-II, scan number 455) (Jansson et al., 1976). The formation of this product has also been reported by Rolf et al. (1985) and appears to be due to traces of water in the trifluoroacetic acid used during the reductive cleavage. Two other minor artifacts, with absolute retention times of 20.47/20.60 (relative to $C_{16}H_{34}$, 1.45/1.46), were also observed in the gas chromatography/mass spectrometry traces (S-I, scan numbers 451 and 455; S-II, scan numbers 587 and 591). They remain uncharacterized but must be related to the *N*-acetylneuraminic acid residue because they were also found when applying the methodology to *N*-acetylneuraminic acid and to the model compound, neuraminyllactose.

Integration of gas chromatographic peak areas (FID detector) of the reductively cleaved products of permethylated S-I and S-II provided a rough estimate of the number of individual sugar residues in each oligosaccharide. The molar ratios of 1,5-anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol:1,4,5-tri-*O*-methyl-3,6-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol:terminal *N*-acetylneuraminic acid were 37:22:41 for S-I and 26:26:48 for S-II. These values support the reported compositions for S-I and S-II (Table I).

Proposed Structures of rSGP-4 Oligosaccharides. On the basis of the data obtained for the isolated oligosaccharide units, the structure of S-I is NeuAcα2→3Galβ1→3(NeuAcα2→3Galβ1→4GlcNAcβ1→6)GalNAc-ol and of S-II is NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAc-ol. S-III consists of two isomers of a trisaccharide with sialic acid attached to either C-3 of galactose or C-6 of *N*-acetylgalactosaminitol in the Galβ1→3GalNAc-ol disaccharide.

Discussion

The rat erythrocyte sialoglycoproteins have been shown in this study to contain several types of oligosaccharide chains that are attached to serine and threonine residues of the polypeptide. The M_r 19000 glycoprotein, rSGP-4, contains only O-glycosidically linked oligosaccharide units as suggested earlier from a study of its composition that indicated a lack of mannose (Edge & Weber, 1980, 1981).

Since a single *N*-acetylgalactosamine was found per oligosaccharide chain, it can be calculated that there are 11 O-linked carbohydrate units in rSGP-4 on the basis of its previously reported composition and molecular weight (Edge & Weber, 1981). The molar distribution of these carbohydrate units in rSGP-4 would be approximately 2 of S-I, 7 of S-II,

and 2 of S-III as estimated by the ratio of formaldehyde released from C-9 of *N*-acetylneuraminic acid and C-6 of *N*-acetylgalactosaminitol upon periodate oxidation. The two S-I oligosaccharides thus account for the two residues of glucosamine found per mole of rSGP-4 (Edge & Weber, 1981).

The presence of *N*-acetylglucosamine in an O-glycosidically linked oligosaccharide of a cell surface glycoprotein has now been described in several cell types (Chandrasekaran & Davidson, 1979; Van den Eijnden et al., 1979; Funakoshi & Yamashina, 1982; Hull et al., 1984). A 3,6-branched *N*-acetygalactosamine core, such as that demonstrated here for S-I from the rat erythrocyte sialoglycoproteins, has also been observed in O-glycosidically linked oligosaccharides from a variant of human glycophorin (Blumenfeld et al., 1981); similar carbohydrate units have been isolated from a platelet glycoprotein (Tsuji et al., 1983) and from cell surface glycoproteins of various tumors (Funakoshi & Yamashina, 1982; Hull et al., 1984). In each of these cases the core *N*-acetylgalactosamine branch points consisted of a 3-linked galactose and a 6-linked *N*-acetylglucosamine. Such structures have been known for some time to occur in mucins and blood group glycoproteins (Lloyd et al., 1968; Oates et al., 1974; Hounsell et al., 1980; Akiyama et al., 1984). The structures of the tri- and tetrasaccharides demonstrated in this study for the rat sialoglycoproteins have been described previously in a variety of secreted and cell surface glycoproteins including human glycophorin (Thomas & Winzler, 1969; Spiro & Bhoyroo, 1974).

The above-noted similarity of the *N*-acetylglucosamine-containing branched oligosaccharide (S-I) to certain mucin-derived O-glycosidically linked oligosaccharides is only one of several notable similarities between this cell surface glycoprotein and secreted mucins. The O-glycosyl linkage is characteristic of mucins and is the only type found in the glycoprotein rSGP-4. The high degree of glycosylation (68% carbohydrate) demonstrated in rSGP-4 (Edge & Weber, 1981) is also typical for mucins, as is the low content of aromatic and sulfur-containing amino acids and preponderance of serine, threonine, proline, alanine, and glycine (Moschera & Pigman, 1975; Hagopian & Eylar, 1968; Hill et al., 1977). These similarities may relate to the structural requirements for glycosylation at serine and threonine residues which appears to occur in regions of the polypeptide that are rich in serine, threonine, and proline (Hill et al., 1977; Young et al., 1979), although no specific primary structure analogous to the Asn-X-Thr(Ser) sequence for N-glycosylation (Eylar, 1965; Hanover & Lennarz, 1981) has been found.

The erythrocyte sialoglycoproteins are thought to play a central role in modulating the life cycle of the rat red blood cell. They undergo well-defined changes in surface distribution during erythropoiesis (Skutelsky & Farquhar, 1976), and their oligosaccharide units have been implicated in the process of clearance of aged erythrocytes from the circulation (Gregoriadis et al., 1974; Aminoff et al., 1977). It is noteworthy that several different carbohydrate structures are present at the cell surface of the rat erythrocyte; moreover, upon removal of sialic acid from these glycoproteins, a step that leads to their recognition and clearance by liver Kupffer and spleen cells (Aminoff et al., 1977), a variety of underlying sugar sequence are exposed.

Registry No. S-I, 81873-60-1; S-II, 68366-20-1; S-III (isomer 1), 68366-21-2; S-III (isomer 2), 68314-59-0.

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Self-Association and Active Enzyme Forms of *Naja naja naja* and *Crotalus atrox* Phospholipase A_2 Studied by Analytical Ultracentrifugation†

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ABSTRACT: The dimerization of phospholipase A_2 ($PLPA_2$) from *Naja naja naja* (Pakistani cobra) and *Crotalus atrox* (Western Diamondback rattlesnake) has been studied from pH 2.5 to 11 at 20 °C in 1 mM $CaCl_2$, 0.21 M ionic strength. For the *C. atrox* enzyme, it was found necessary to use a combination of sedimentation equilibrium and fluorescence yield data to analyze the association. Sedimentation equilibrium in the analytical ultracentrifuge sufficed for the study of the *N. naja* $PLPA_2$. In the region of enzymatic activity at pH 8, the dimerization association constants found were $k_2 = 2.8 \times 10^6$ L/mol and $k_2 = 6.9 \times 10^4$ L/mol for the *C. atrox* and *N. naja* enzymes, respectively. Analytical linked functions are presented which describe the data. Because the associations are linked to Ca^{2+} as well as the hydrogen ion, no attempt was made to interpret the ionization of residues in terms of the molecular structure. Active-enzyme sedimentation velocity experiments have been used to study the relation between enzymatic activity and association for both the *C. atrox* and *N. naja* enzymes. The substrate 1,2-dibutyryl-*sn*-glycero-3-phosphocholine (diC4PC) did not dissociate the *C. atrox* $PLPA_2$. The substrate 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (diC6PC) at 7.5 mM dissociated the *C. atrox* $PLPA_2$ when monitored either as the active enzyme or as the Sr^{2+}-inhibited enzyme. At low enzyme concentrations, 40 mM diC4PC had no effect on *N. naja* $PLPA_2$ dimerization. However, the sedimentation coefficients observed at enzyme concentrations above 0.2 mg/mL in active-enzyme sedimentation velocity experiments were larger than the values predicted from the thermodynamic studies. Sedimentation coefficients observed for the *N. naja* $PLPA_2$ acting on diC6PC were larger than those of the monomeric protein, which was the form layered on this substrate. The dissociation of the *C. atrox* $PLPA_2$ effected by diC6PC was analyzed by the thermodynamics of association and the kinetic Michaelis constant. The analysis suffices to account for the observed sedimentation coefficient. The sedimentation behavior of the *N. naja* $PLPA_2$ acting on diC6PC substrate was analyzed in terms of a protein–lipid complex. With this model, 68 ± 16 phospholipid molecules per protein monomer were determined. It is proposed that this enzyme has two micelle nucleation sites per monomer. These putative sites promote micelle formation of the substrate on the enzyme below the critical micelle concentration of the lipid alone.

Considerable controversy has been generated over the years concerning the active enzymatic species of the phospholipase A_2 enzymes. At the current time, it is accepted [see review by Verheij et al. (1981)] that *Crotalus adamanteus* phospholipase A_2 hydrolyzes small nonmicellar substrates as the dimeric protein. For the cobra (*Naja*) venom enzymes, a variety of associated states of the protein have been proposed, but the connection between association and activity is by no means clear.

We have been examining the self-association of both the *Crotalus atrox* and *Naja naja naja* phospholipase A_2 enzymes by sedimentation equilibrium in the analytical ultracentrifuge and by fluorescence studies. It is natural for us to be interested in the relation between the dimerization in the absence of substrate and the enzymatic activity of the proteins. The absence of thermodynamic linkage between association and substrate binding, as was found for the saccharide binding by concanavalin A (Senear & Teller, 1981b), would mean that our thermodynamic studies are applicable to the enzymology as well as the protein structure. On the other hand, linkage of the substrate binding and turnover to the dimerization is of direct interest to structure–function relationships of these enzymes.

One particularly puzzling aspect of the enzymology of *Crotalus atrox* phospholipase A_2 is that the enzyme is gen-

† This work was supported by National Institutes of Health Grant GM 13401.

0006-2960/86/0425-8024$01.50/0