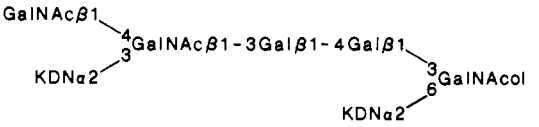
Isolation and Structure of a Novel Deaminated Neuraminic Acid Containing Oligosaccharide Chain Present in Rainbow Trout Egg Polysialoglycoprotein

Mariko Iwasaki,[‡] Sadako Inoue,*[,‡] Daita Nadano,[§] and Yasuo Inoue*[,§]

School of Pharmaceutical Sciences, Showa University, Hatanodai, Tokyo 142, Japan, and Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

Received June 6, 1986; Revised Manuscript Received September 11, 1986

ABSTRACT: A novel acidic oligosaccharide alditol containing a deaminated neuraminic acid derivative was isolated from polysialoglycoprotein of rainbow trout eggs on alkaline borohydride treatment. From the structural studies including gas–liquid chromatography of monosaccharide derivatives, methylation analysis, and 400-MHz ^{1}H NMR spectroscopy, we propose the structure



where KDN = 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid, for a minor but integral sugar chain present in polysialoglycoprotein. This provides the first identification of a natural occurrence of deaminated sialic acid residues found as an integral part of oligosaccharide chains in a glycoprotein.

The first polysialoglycoproteins (PSGP;[1] molecular mass about 200 kDa) from the unfertilized eggs of the Salmonidae fish family were described in 1978 (Inoue & Iwasaki, 1978). The previous structural studies established the presence of oligo(poly)sialyl groups and novel core oligosaccharide chains in PSGP (Inoue & Iwasaki, 1980; Inoue et al., 1981; Nomoto et al., 1982; Shimamura et al., 1983; Iwasaki et al., 1984a,b; Kitajima et al., 1984; Shimamura et al., 1984; Iwasaki & Inoue, 1985). We have recently arrived at the conclusion that the protein backbone (molecular mass about 30 kDa) of the rainbow trout egg PSGP constitues sequences of tridecapeptides that are tandemly repeating, and the main structural features of the PSGP have been outlined by Kitajima et al. (1986). Furthermore, our recent studies have revealed that this novel class of sialic acid rich glycoproteins (PSGP) are primarily localized in the cortical alveoli of unfertilized eggs of Salmonidae fish and undergo a rapid and dramatic proteolytic depolymerization to 9-kDa low-molecular-mass PSGP upon fertilization (Inoue & Inoue, 1986). Most recently, we have also demonstrated the occurrence in rainbow trout egg PSGP of an unknown acidic sugar component besides sialic acids originally designated "SiaX" [see Kitajima et al. (1986)], and the exact chemical structure of "SiaX" was determined as 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (KDN; see Figure 1) by chemical, enzymatic and high-resolution ^{1}H NMR spectroscopic comparison of the naturally occurring component with authentic product of chemical synthesis (Nadano et al., 1986). KDN represents the first naturally occurring deaminated sialic acid residue in biological material.

This paper describes the identification of a KDN-containing oligosaccharide alditol isolated from a mixture of acidic oligosaccharide alditols released by alkaline borohydride treatment of PSGP.

Materials and Methods

Materials. PSGP was prepared from the unfertilized eggs of *Salmo gairdneri* as previously described (Inoue & Matsumura, 1979). Sialidase from *Streptococcus* sp. IID6646 was obtained from Seikagaku Kogyo Co., Tokyo. D_2O of 99.85% and 99.95% purity was obtained from Commissariat a l'Enerqie Atomique (CEA).

Thin-Layer Chromatography. Purity of oligosaccharides was monitored by TLC on silica gel 60 plates (Merck, Darmstadt, West Germany) as described previously (Iwasaki & Inoue, 1985). Solvent I (1-propanol/concentrated NH_4OH/H_2O, 6/1/2.5 by volume) was used as a developer.

Carbohydrate Composition Analysis. The molar ratios of component sugars were determined by GLC (Nomoto et al., 1982). Free sialic acid released by hydrolysis was determined by the modified thiobarbituric acid method (Iwasaki et al., 1984a) at 550 nm. Neutral sugar was determined by the phenol–sulfuric acid method (Dubois et al., 1956). The molar relative response factor for KDN in GLC analysis was based on the standard compound KDNα2–8NeuGc obtained from the PSGP (Nadano et al., 1986).

Methylation Analysis. Permethylation was carried out as described previously [Iwasaki & Inoue, 1985; cf. also Ciucanu and Kerek (1984)]. The partially methylated alditol acetates were identified by GLC–mass spectrometry with a JEOL JMS-300 mass spectrometer–JGC-20KP gas chromatograph.

^{1}H NMR Spectroscopy. Oligosaccharides were repeatedly treated with D_2O (99.85%), with lyophilization between treatments, and finally dissolved in D_2O (99.95%). The 400-MHz spectra were recorded on a Bruker WM 400 spectrometer at appropriate temperatures. Chemical shifts were measured to internal 2-methyl-2-propanol (1.237 ppm)

‡Showa University.
§University of Tokyo.

[1] Abbreviations: PSGP, polysialoglycoprotein; KDN, 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid or 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid [KDN corresponds to "sialosonic acid" (SiaA) in the nomenclature of Scott et al. (1982)]; NeuGc, *N*-glycolyl-D-neuraminic acid; TBA, thiobarbituric acid; SRO, GalNAcβ1–4[NeuGcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3GalNAcol; T3L, GalNAcβ1–4[NeuGcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3[NeuGcα2–6]GalNAcol; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; ^{1}H NMR, proton nuclear magnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/87/0426-1452$01.50/0

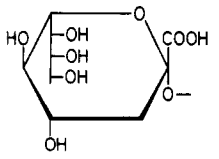


FIGURE 1: Structure of the KDN (=3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid) residue present in rainbow trout egg PSGP.

and expressed relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

RESULTS

Isolation and Purification of the KDN-Containing Acidic Oligosaccharide Alditol, Long-Cored KDN-Containing Oligosaccharide, 1-b1-0.1M. Sialooligosaccharide alditols released by alkaline borohydride (1 M $NaBH_4$/0.1 M NaOH) treatment (Inoue & Matsumura, 1979) of PSGP (250 mg) of *Salmo gairdneri* were first subjected to Sephadex G-25 column (2 × 122 cm) chromatography. When fractions were analyzed for both sialic acid and neutral sugar by the TBA and phenol–sulfuric acid methods, respectively, two partially resolved peaks were obtained (not shown). The material in the first major peak (140 mg) was fractionated on a DEAE-Sephadex A-25 column with a linear gradient of NaCl concentration (Figure 2A). The column fractions were assayed for sialic acid. The A_{550} pattern corresponds roughly to the elution pattern shown in Figure 1 of the previous paper (Nomoto et al., 1982). When the individual fractions (peak 1–5) were analyzed by GLC, the KDN-specific peak [cf. Nadano et al. (1986)] was detected only for fraction 1-b and, to a lesser extent, for fraction 1-c. Further search for KDN-containing higher acidic oligosaccharides seemed desirable, but in this paper emphasis has been put on the structural analysis of the prevalent KDN-containing oligosaccharide alditols. Thus, the contents of the tubes under peak 1 were pooled, concentrated in vacuo, desalted by passage through a Sephadex G-10 column (1.5 × 69 cm), and rechromatographed on the same DEAE-Sephadex A-25 column (inset in Figure 2A). Incompletely resolved peaks and a terminal shoulder, designated 1-a, 1-b, and 1-c, were pooled separately and lyophilized after desalting (1-a, 2.8 mg; 1-b, 1.9 mg; 1-c, 1.0 mg). Further purification of the subfractions, 1-a and 1-b, was achieved by column chromatography on Bio-Rad AG 1-X2 (Cl^-) (Figure 2B). Only fraction 1-b1 was found to contain an appreciable amount of KDN [Fuc/Gal/GalNAc/GalNAcol/KDN/NeuGc = 0.32/1.60/1.34/1.00/1.26/0.63 (mol/mol); cf. for fraction 1-b2, Gal/GalNAc/GalNAcol/KDN/NeuGc = 1.66/1.09/1.00/0.23/1.58 (mol/mol)] and was shown to be a mixture of at least two components when examined by TLC (vide infra). Fraction 1-b1 was therefore digested with *Streptococcus* sialidase in 0.1 M KH_2PO_4 buffer (pH 6.5, 0.2 mL), for 24 h at 37 °C under toluene. Enzymatic digestion was repeated twice, and the separation of the sialidase-resistant 1-b1 fraction from NeuGc and other minor hydrolysis products could be effected by DEAE-Sephadex A-25 column (1.5 × 4 cm) chromatography. The column was first eluted with 20 mL of 0.05 M NaCl and then with 30 mL of 0.1 M NaCl. A test of homogeneity was carried out by TLC. The fraction eluted with 0.1 M NaCl showed only a single spot (orcinol spray; Schauer, 1978) on TLC and is denoted as the 1-b1-0.1M fraction. Materials denoted as 1-a1 [Gal/GalNAc/GalNAcol/NeuGc = 1.79/1.81/1.0/2.17 (mol/mol)] and 1-a2 [Fuc/Gal/GalNAc/GalNAcol/NeuGc = 0.93/1.80/1.21/1.0/1.75 (mol/mol)] were found to be homogeneous, and their structures are respectively proposed to be identical with those recently es-

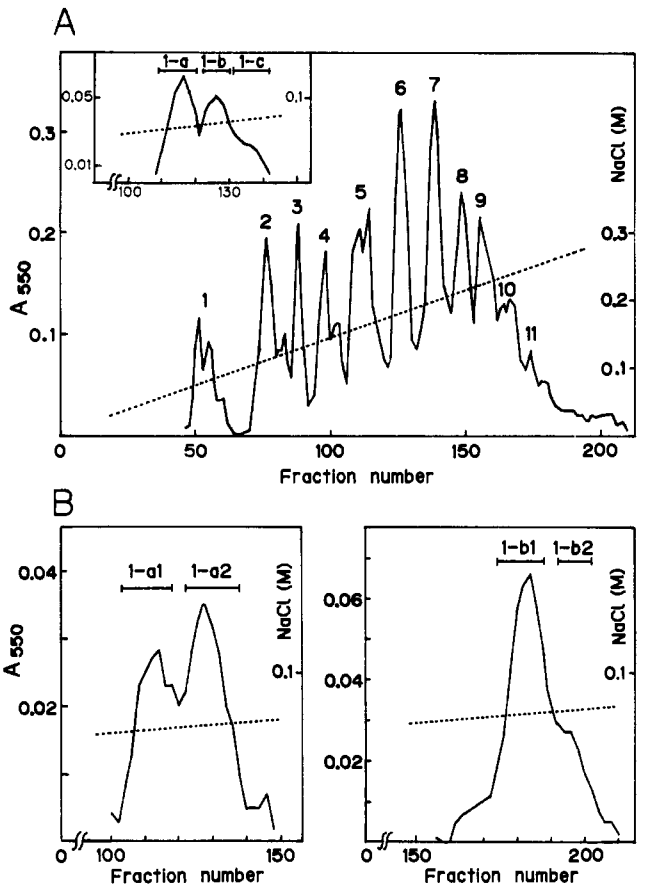


FIGURE 2: (a) Fractionation of acidic oligosaccharide alditols (140 mg) derived from rainbow trout egg PSGP (250 mg) by ion-exchange chromatography on a DEAE-Sephadex A-25 column (0.9 × 96 cm). The column was developed with 100 mL of equilibration buffer (0.02 M Tris-HCl, pH 8) and then with the following linear gradients: 800 mL of 0 M NaCl/0.02 M Tris-HCl, pH 8.0, starting buffer and 800 mL of 0.4 M NaCl/0.02 M Tris-HCl, pH 8.0. The columns were monitored manually as described in the text. Fractions containing 6 mL were collected. (Inset) DEAE-Sephadex A-25 rechromatogram of fraction 1 obtained as indicated. The column (0.9 × 96 cm) was eluted with a 800-mL linear gradient of NaCl (0–0.1 M)/0.02 M Tris-HCl buffer, pH 8.0. Material was collected in 4-mL fractions. The bars indicate those fractions pooled to give 1-a, 1-b, and 1-c. (B) Elution patterns from the column chromatography of 1-a and 1-b. The column consisted of Bio-Rad AG 1-X2 (Cl^- form), 200–400 mesh (1.5 × 55 cm). Elution was effected in each case by an 800-mL linear gradient of NaCl (0.05–0.09 M) and then with 0.1 M NaCl. Fractions were collected every 4 mL and pooled as indicated by the bars.

tablished for T3L (Iwasaki et al., 1984a; Kitajima et al., 1984) and T3F (Iwasaki et al. 1984b) [or S6-1 (Shimamura et al., 1984)], isolated from rainbow trout and Pacific salmon egg PSGPs, by sugar analysis and comparison of TLC behavior:

GalNAcβ1-4[NeuGcα2-3]GalNAcβ1-3Galβ1-4Galβ1-3[NeuGcα2-6]GalNAcol
1-a1

Fucα1-3GalNAcβ1-3Galβ1-4Galβ1-3[NeuGcα2-8NeuGcα2-6]GalNAcol
1-a2

Composition and Methylation Analysis of the KDN-Containing Oligosaccharide Alditol, 1-b1-0.1M. Part of the oligosaccharide alditol, the 1-b1-0.1M fraction, was used for sugar analysis, and the remaining materials served for linkage analysis (methylation analysis) and 400-MHz ^1H NMR spectroscopy. The molar ratios of the constituent sugars are Gal:GalNAc:KDN:GalNAcol = 2:2:2:1 for the purified 1-b1-0.1M. The identities of the GLC peaks obtained for the mixture of partially methylated alditol acetates derived from 1-b1-0.1M were confirmed by GLC–MS. The following partially methylated alditol acetates were identified: 2,3,6-*O*-Me_3-1,4,5-*O*-Ac_3-Galol; 2,4,6-*O*-Me_3-1,3,5-*O*-Ac_3-Galol;

2-*N*-Me-3,4,6-*O*-Me_3-1,5-*O*-Ac_2-GalNAcol; 2-*N*-Me-6-*O*-Me-1,3,4,5-*O*-Ac_4-GalNAcol; 2-*N*-Me-1,4,5-*O*-Me_3-3,6-*O*-Ac_2-GalNAcol. The composition of 1-b1-0.1M indicated the presence of two KDN residues and no NeuGc residue, and thus the results of methylation analysis showed that these KDN residues occupied terminal positions in the oligosaccharide.

Sequence of 1-b1-0.1M. 1-b1-0.1M and SRO [GalNAcβ1–4[NeuGcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3GalNAcol; a unique sialidase-resistant oligosaccharide alditol of which the structure was determined unambiguously by Iwasaki et al. (1984a) and Kitajima et al. (1984)] contained the same numbers of constituent Gal, GalNAc, and GalNAcol residues, which suggests an identical structure for the core pentasaccharide alditol. Lack of material prevented further degradative work such as Smith degradation and hydrazinolysis–nitrous deamination conducted on SRO and asialo-SRO in a previous study (Iwasaki et al., 1984). There are at least six alternative structures for 1-b1-0.1M that could accommodate the results of methylation analysis. Pending additional work, our decision in favor of the structure GalNAc(1–4)-[KDN(2–3)]GalNAc(1–3)Gal(1–4)Gal(1–3)[KDN(2–6)]-GalNAcol for 1-b1-0.1M was made through a consideration of the five distinct consensus sequences of oligo(poly)sialylglycan units in PSGP molecules found in a wide variety of Salmonidae fish eggs (Iwasaki & Inoue, 1985): (1) Galβ1–3GalNAc*; (2) Galβ1–4Galβ1–3GalNAc*; (3) GalNAcβ1–3Galβ1–4Galβ1–3GalNAc*; (4) Fucα1–3GalNAcβ1–3Galβ1–4Galβ1–3GalNAc*; (5) GalNAc β1–4[NeuAcylα2–3]GalNAcβ1–3Galβ1–4Galβ1–3GalNAc*. The asterisk (*) indicates the proximal sugar residues to which oligo(poly)sialic acid chains are attached through α2–6 linkages. Supportive evidence is presented from its high-resolution ^{1}H NMR spectral measurement (vide infra). In the low-field anomeric region and in the up-field region for *N*-acetyl proton signals, all these corresponding signals appear essentially at the same positions as are observed in the spectra of T3L (= GalNAcβ1–4[NeuGcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3-[NeuGcα2–6]GalNAcol) and SRO (Kitajima et al., 1984). The only definitive difference in the ^{1}H NMR spectrum of 1-b1-0.1M when compared to those of T3L and SRO is the absence of the *N*-glycolyl methylene proton signal(s) at about 4.1 ppm, which is in accordance with the fact that no NeuGc residue is present in 1-b1-0.1M. To our satisfaction, we were able to confirm that the core pentasaccharide sequence of 1-b1-0.1M, T3L, and SRO was identical.

A combination of the overall evidence offers convincing proof of the structural formulation of 1-b1-0.1M acidic oligosaccharide as

GalNAcl–4[KDN2–3]GalNAcl–3Gall–4Gall–3[KDN2–6]GalNAcol
I

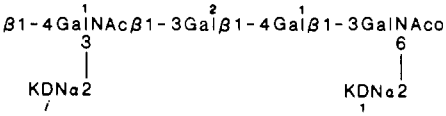
We have further substantiated the assignment of the anomeric configurations of the glycosidic linkages in 1-b1-0.1M from 400-MHz ^{1}H NMR spectral analysis.

400-MHz ^{1}H NMR Spectroscopy. The ^{1}H NMR spectrum of 1-b1-0.1M as shown in Figure 3 indicated the presence of four proton signals in the anomeric region of the spectrum (4.49 ppm, 1 H; 4.59 ppm, 1 H; 4.80 ppm, 2 H), and all of these resonances can be assigned to individual protons solely on the basis of previously published NMR data on GalNAcβ1–4[NeuGcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3GalNAcol and GalNAcβ1–4[NeuGcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3[NeuGcα2–6]GalNAcol, designated as SRO and T3L, respectively, by Kitajima et al. (1984). The first two signals with $J_{1,2}$ = 7.9 and 8.5 Hz are attributed to β-linked anomeric protons of Gal[1] and Gal[2] residues, respectively (see Table I for the residue numbering); the last unresolved signals with $J_{1,2}$ = 7.9 Hz, measured at 60 °C, are reminiscent of H-1_{eq} of the β-linked terminal and penultimate GalNAc residues. The NMR peaks at 2.48 and 2.66 ppm (one proton each, doublet of doublets, J_{AB} = 12 Hz, J_{AX} = 4 Hz) are those derived from the C-3 methylene groups in KDN (KDN[*i*]–H-3_{eq} and KDN[1]–H-3_{eq}, respectively). Likewise, the pair of doublets (J_{BX} = 12 Hz) at 1.79 ppm (KDN[*i*]–H-3_{ax}) and 1.66 ppm (KDN[1]–H-3_{ax}) were assigned. Each of these peaks assignable to the KDN C-3 methylene protons corresponds to the respective peak of H-3's in T3L (Table I). It should be noted that an examination of the literature suggests that analysis by high-field ^{1}H NMR spectroscopy sometimes with the two-dimensional *J*-resolved ^{1}H NMR spectroscopy technique has been increasingly applied to reduced O-linked oligosaccharides [e.g., Van Halbeek (1984), Donald and Feeney (1986), Mutsaers et al. (1986), and Nasir-Ud-Din et al. (1986)], and therefore, the resonance observed downfield at about 4.0 ppm in the spectrum (Figure 3) may be assignable to the specific protons on the basis of comparisons with the reported spectra of different oligosaccharide alditols. However, while oligosaccharide alditols comprising Gal, GlcNAc, and GalNAcol exhibited rather simple spectra in this 4-ppm region [GalNAcol H-3, GalNAcol H-5, Gal H-3: Van Halbeek et al. (1982) and Mutsaers et al. (1986)], those having a GalNAc residue(s) as their constituent sugar tend to reveal a rather complex nature in the spectra near 4 ppm (Van Halbeek et al., 1981; Nasir-Ud-Din et al., 1986), so that qualitative comparison hardly constitutes solid proof of the accurate assignments of the signals at the 4.0–4.2-ppm region. In this study, insufficient material only

Table I: Proton (Anomeric Protons, H-3 of KDN and NeuGc Residues, *N*-Acetyl Methyl Protons, and H-2 of GalNAcol) Chemical Shifts and Spin-Coupling Constants of 1-b1-0.1M and T3L[a]

proton	1-b1-0.1M	T3L[b]
H-1 of		
Gal[1]	4.49 [7.9]	4.50
Gal[2]	4.59 [8.5]	4.60
GalNAc[1]	(4.80) [(7.9)]	
GalNAc[2]	(4.80) [(7.9)]	
H-3_{eq} of		
KDN[*i*]	2.48 [J_{AB} = 12; J_{AX} = 4.0]	
KDN[1]	2.66 [J_{AB} = 12; J_{AX} = 4.1]	
NeuGc[1]		2.75
NeuGc[*i*]		2.56
H_{ax} of		
KDN[*i*]	1.79 [J_{BX} = 12]	
KDN[1]	1.66 [J_{BX} = 12]	
NeuGc[1]		1.71
NeuGc[*i*]		1.85
NAc protons of		
GalNAcol	2.06	2.08
GalNAc[1]	2.00	2.01
GalNAc[2]	2.04	2.05
H-2 of		
GalNAcol	4.37	4.37

[a] Chemical shifts are measured in D_2O at 23 °C and are expressed in ppm relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Spin-coupling constants are given in Hz in brackets. Those in parentheses are the corresponding values measured at 60 °C. The residue numbering for 1-b1-0.1M (II) is the same as that used by Kitajima et al. (1984):



[b] Data taken from Kitajima et al. (1984).

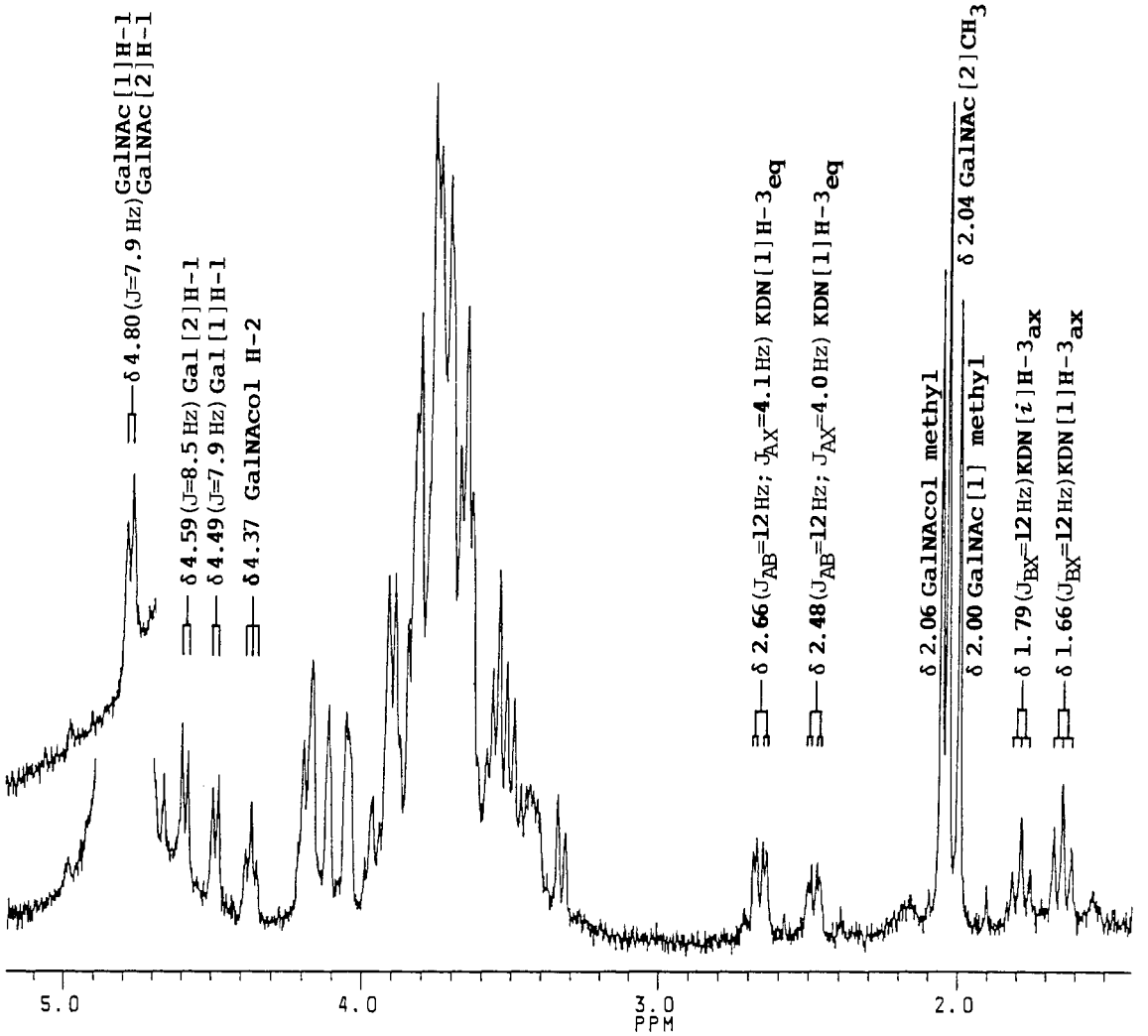
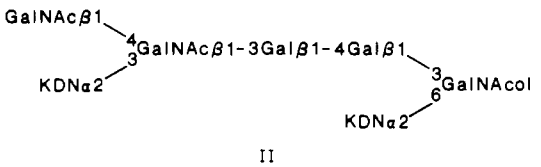


FIGURE 3: The 400-MHz ^1H NMR spectrum of 1-b1-0.1M in D_2O at 23 °C. The 4.7–5.2-ppm region of the spectrum at 60 °C is also shown particularly because in the 23 °C spectrum the anomeric proton resonances for the terminal and penultimate GalNAc residues are hidden under the HDO signal.

allowed us to measure ^1H NMR spectra at about 100 nmol of oligosaccharide alditols, but the result shown in Figure 3 does suggest that 1-b1-0.1M has the indicated structure. A summary of all of the assignments of 1-b1-0.1M is presented in Table I together with those for T3L. Because the anomeric configurations of the linkages of KDN residues were considered in the preceding paper (Nadano et al., 1986) (the ^1H NMR signals of both H-3_{eq} and H-3_{ax} protons in KDN were correlated with the anomeric configuration at the C-2 atom), it was not considered necessary to investigate this aspect of the structure of 1-b1-0.1M. Structure II is thus derived and is fully corroborated by the above NMR data.



II

Distribution of KDN in PSGP from Salmonid Fishes. When *Salmo*, *Oncorhynchus*, and *Salvelinus* fish egg PSGPs were analyzed for carbohydrate composition by GLC, small unknown peaks having identical retention time with that for KDN were detected. The amount of KDN in PSGP from the eggs of several species of salmonid fish was estimated by GLC analysis, and the results are shown in Table II. KDN was not detected in a specimen of *Oncorhynchus keta* egg PSGP we used. Nevertheless, KDN has been detected in the PSGP preparations from a rather wide variety of salminid fish species (Table II). Further research will be required to understand the possible function of the KDN moiety in PSGP.

Table II: The KDN Present in PSGP from the Eggs of Salmonidae Fishes[a]

Salmonidae fish	KDN/Neu5Acyl (%)
Salmo gairdneri	6.7
Salmo trutta fario	13[b]
Oncorhynchus keta	<0.1
Oncorhynchus masou ishikawai	1.3
Oncorhynchus nerka adonis	3.4
Salvelinus leucomaenis	2.0
Salvelinus namaycush	9.6[b]
Salvelinus fontinalis	4.1[b]

[a] The sialic acid (Neu5Acyl) contents were based on the resorcinol method, and the KDN was determined by GLC. KDN/Neu5Acyl is a molar ratio. [b] Determined by Dr. I. Tazawa in our labboratory.

DISCUSSION

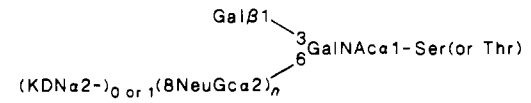
During the last 2 decades of research on modified sialic acids in glycoconjugates, the most important achievements were (a) the discovery of more than 30 modified sialic acids, (b) the elucidation of their structures and positions in specific glycoconjugates, and (c) the clarification of their biosynthetic routes and possible regulatory functions (Corfield & Schauer, 1982; Schauer, 1983). We found a uniquely modified sialic

acid, deaminated sialic acid, for the first time in PSGP from rainbow trout eggs and established its structure as 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (KDN; see Figure 1), and the experiment showed that no hydrolysis can be detected with the bacterial sialidases used (Nadano et al., 1986). The previous study has also established that the KDN residues are located at the nonreducing ends of oligo(poly)sialyl groups in the form of KDNα2–(8NeuGcα2–)$_n$-, thereby protecting the oligo(poly)sialyl groups from hydrolysis by sialidases (Nadano et al., 1986).

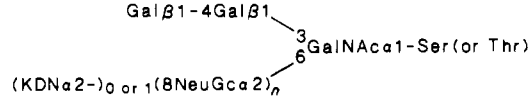
In this study we have further revealed additional modes of the occurrence of the KDN residues other than as the "capping group" in PSGP of rainbow trout eggs by isolating a KDN-containing oligosaccharide alditol. Results from our laboratories have demonstrated the existence of five different oligo(poly)sialylglycan units in PSGP (Nomoto et al., 1982; Iwasaki et al., 1984a,b; Kitajima et al., 1984; Iwasaki & Inoue, 1985). The structure (II) now elucidated for a KDN-containing oligosaccharide alditol, 1-b1-0.1M, is classified as a KDN-containing analogue of the typical long-core units (Iwasaki et al., 1984a,b; Kitajima et al., 1984). In both SRO (=GalNAcβ1–4[NeuGcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3GalNAcol) (Iwasaki et al., 1984a; Kitajima et al., 1984) and the NeuAc analogue of SRO, i.e., GalNAcβ1–4[NeuAcα2–3]GalNAcβ1–3Galβ1–4Galβ1–3GalNAcol (Iwasaki & Inoue, 1985), the presence of a GalNAc residue β1–4 linked to the penultimate GalNAc residue to which the sialic acid is linked α2–3 is unfavorable for the action of sialidase due to steric hindrance exerted by the terminal GalNAc residue. The deaminated neuraminic acid residue (KDN) was found to be intrinsically inactive to sialidase (Nadano et al., 1986), and the KDN-containing oligosaccharides tested, namely, KDNα2–8(NeuGcα2–8)$_n$ (n = 1–7) and GalNAcβ1–4[KDNα2–3]GalNAcβ1–3Galβ1–4Galβ1–3[KDNα2–6]GalNAcol (1-b1-0.1M), were all inactive as sialidase substrates.

These results, when combined with the previous studies, have demonstrated that the KDN residues occur as the nonreducing terminal groups involved in α2–3, α2–6, or α2–8 linkages in the five distinct types of oligo(poly)sialylglycan units:

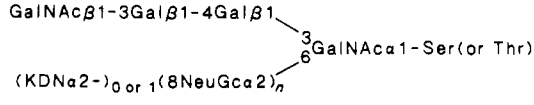
(1) short-core units



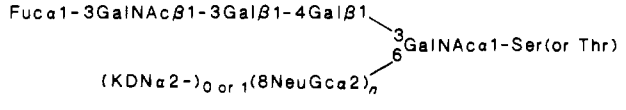
(2) trisaccharide core units



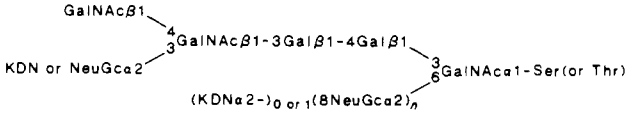
(3) tetrasaccharide core units



(4) fucose-containing core units



(5) long-core units



The KDN residues presumably are placed in these specific locations for definite reasons, but nothing is yet known about their specific functions. Nevertheless, the possible biogenetic sequence for incorporation of a KDN residue(s) into an oligo(poly)sialylglycan chain is presumed as a result of either of the two alternative pathways: (a) conversion to CMP–KDN at the activated CMP-bound sialic acid level and transfer of a KDN residue from CMP–KDN to a 3-OH of the penultimate GalNAc residue in the long-core unit, to a 6-OH of the proximal GalNAc residue, and/or to an 8-OH at the nonreducing terminal NeuGc of an oligo(poly)sialyl group; (b) enzymatic deacylation of the sialic acid residues at nonreducing termini followed by deamination catalyzed by a specific deaminase. It should be noted here that these two alternative mechanisms would imply that the KDN residue once incorporated can never be an acceptor for NeuGc and/or KDN. In PSGP, KDN is thus assumed to act as "chain stopper" in the biosynthetic process, controlling the extent of chain elongation of sialyl groups.

ACKNOWLEDGMENTS

We are indebted to Professors T. Miyazawa and Y. Arata and to S. Endo for their aid in obtaining the ^{1}H NMR spectra.

REFERENCES

Ciucanu, I., & Kerek, F. (1984) *Carbohydr. Res. 131*, 209–217.

Corfield, A. P., & Schauer, R. (1982) in *Sialic Acids: Chemistry, Metabolism and Function* (Schauer, R., Ed.) pp 5–50, Springer-Verlag, New York.

Donald, A. S. R., & Feeney, J. (1986) *Biochem. J. 236*, 821–828.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Inoue, S., & Iwasaki, M. (1978) *Biochem. Biophys. Res. Commun. 83*, 1018–1023.

Inoue, S., & Matsumura, G. (1979) *Carbohydr. Res. 74*, 361–368.

Inoue, S., & Iwasaki, M. (1980) *Biochem. Biophys. Res. Commun. 93*, 162–165.

Inoue, S., & Inoue, Y. (1986) *J. Biol. Chem. 261*, 5256–5261.

Inoue, S., Iwasaki, M., & Matsumura, G. (1981) *Biochem. Biophys. Res. Commun. 102*, 1295–1301.

Iwasaki, M., & Inoue, S. (1985) *Glycoconjugate J. 2*, 209–228.

Iwasaki, M., Inoue, S., Kitajima, K., Nomoto, H., & Inoue, Y. (1984a) *Biochemistry 23*, 305–310.

Iwasaki, M., Nomoto, H., Kitajima, K., Inoue, S., & Inoue, Y. (1984b) *Biochem. Int. 8*, 573–579.

Kitajima, K., Nomoto, H., Inoue, Y., Iwasaki, M., & Inoue, S. (1984) *Biochemistry 23*, 310–316.

Kitajima, K., Inoue, Y., & Inoue, S. (1986) *J. Biol. Chem. 261*, 5262–5269.

Mutsaers, J. H. G. M., Van Halbeek, H., Vliegenthart, J. F. G., Wu, A. M., & Kabat, E. A. (1986) *Eur. J. Biochem. 157*, 139–146.

Nadano, D., Iwasaki, M., Endo, S., Kitajima, K. Inoue, S., & Inoue, Y. (1986) *J. Biol. Chem. 261*, 11550–11557.

Nasir-Ud-Din, Jeanloz, R. W., Lamblin, G., Roussel, P., Van Halbeek, H., Mutsaers, J. H. G. M., & Vliegenthart, J. F. G. (1986) *J. Biol. Chem. 261*, 1992–1997.

Nomoto, H., Iwasaki, M., Endo, T., Inoue, S., Inoue, Y., & Matsumura, G. (1982) *Arch. Biochem. Biophys. 218*, 335–341.

Schauer, R. (1978) *Methods Enzymol. 50*, 64–89.

Schauer, R. (1983) *Biochem. Soc. Trans. 11*, 270–271.

Scott, J. E., Yamashina, I., & Jeanloz, R. W. (1982) *Biochem. J. 207*, 367–368.

Shimamura, M., Endo, T., Inoue, Y., & Inoue, S. (1983) *Biochemistry 22*, 959–963.
Shimamura, M., Endo, T., Inoue, Y., Inoue, S., & Kambara, H. (1984) *Biochemistry 23*, 317–322.
Van Halbeek, H. (1984) *Biochem. Soc. Trans. 12*, 601–605.
Van Halbeek, H., Dorland, L., Haverkamp, J., Veldink, G. A., Vliegenthart, J. F. G., Fournet, B., Ricart, G., Monteuil, J., Gathmann, W. D., & Aminoff, D. (1981) *Eur. J. Biochem. 118*, 487–495.
Van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Hull, W. E., Lamblin, G., Lhermitte, M., Boersma, A., & Roussel, P. (1982) *Eur. J. Biochem. 127*, 7–20.

Homologues of the Human C and A Apolipoproteins in the *Macaca fascicularis* (Cynomolgus) Monkey†

Peter N. Herbert,*,‡ Linda L. Bausserman,‡ Karen M. Lynch,‡ Ann L. Saritelli,‡ Mark A. Kantor,‡ Robert J. Nicolosi,§ and Richard S. Shulman‡

Brown University Program-in-Medicine, Department of Medicine, The Miriam Hospital, Providence, Rhode Island 02906, and College of Health Professions, University of Lowell, Lowell, Massachusetts 01854

Received June 25, 1986; Revised Manuscript Received October 28, 1986

ABSTRACT: We used antisera to human A and C apolipoproteins to identify homologues of these proteins among the high-density lipoprotein apoproteins of *Macaca fascicularis* (cynomolgus) monkeys, and NH_2-terminal analysis was used to verify the homology. The NH_2-terminal sequence of the *M. fascicularis* apoA-I is identical with that of another Old World species, *Erythrocebus patas*, and differs from human apoA-I at only 4 of the first 24 residues. *M. fascicularis* apoA-II contains a serine for cysteine replacement at position 6 and is therefore monomeric like the apoA-II from all species below apes. Human and monkey apoA-II are not otherwise different through their first 25 residues. About 20% of *M. fascicularis* apoC-I aligns with human apoC-I through residue 22, and 80% lacks an NH_2-terminal dipeptide. Otherwise, the monkey apoC-I differs from the human protein at only 2 of 25 positions. Two forms of *M. fascicularis* apoC-II were identified. ApoC-II_1 is highly homologous with human apoC-II, whereas an NH_2-terminal hexapeptide is absent from apoC-II_2. ApoC-II_2 was the predominant species, and apoC-II_1 appears to represent a propeptide from which a hexapeptide prosegment is cleaved at a Gln–Asp bond. Both forms of monkey apoC-II are potent activators of lipoprotein lipase. There are two polymorphic forms of *M. fascicularis* apoC-III, and their electrophoretic mobilities become identical after treatment with neuraminidase. Except for a glycine for serine substitution at position 10, the first 15 NH_2-terminal residues of *M. fascicularis* and human apoC-III are the same.

Subhuman primates are widely employed in studies of lipoprotein metabolism, but their apolipoproteins remain incompletely characterized. The A apoproteins of high-density lipoproteins (HDL)[1] have received the most attention (Edelstein et al., 1973, 1976; Blaton et al., 1974, 1977; Mahley et al., 1976), and these have been shown to be highly homologous to their human counterparts (Brewer et al., 1972, 1978).

Little is known of the properties of the lower molecular weight C apolipoproteins in subhuman primates. Parks and Rudel (1979) were unable to isolate a homologue of apoC-I from vervet HDL, and they found apoC-II to differ markedly in amino acid composition and sialic acid content from human apoC-II. They identified only a single homologue of apoC-III, which in humans is found in at least three forms differing in sialic acid content.

We have been using the relatively abundant *Macaca fascicularis* (cynomolgus) species of Old World monkey in studies of lipoprotein metabolism.[2] We undertook these studies to identify and characterize the HDL apolipoproteins in this species, particularly the C apoproteins, and to establish their homology with human HDL proteins. Identification was first based on immunologic cross-reactivity between the monkey and human proteins and was verified by NH_2-terminal sequence analysis in each case.

Materials and Methods

Isolation of Lipoproteins. Sera were obtained at the time of tuberculin testing from the majority of the *M. fascicularis* monkeys in the New England Regional Primate Research Center colony. The animals were not fasting and were sampled throughout the working day. They were permitted unlimited access to monkey chow containing approximately 5% fat. HDL were isolated by preparative ultracentrifugation (Havel et al., 1955) at d = 1.080–1.21 g/mL after sequential removal of the very low density (d = 1.019 g/mL) and low-density lipoproteins (d = 1.063 g/mL). The HDL were recentrifuged

†Supported by NIH Grants HL26156 to L.L.B., HL23792 and HL34637 to R.J.N., and RR00168 and by The R. J. Reynolds Multidisciplinary Program in Cardiovascular Disease and The Miriam Hospital Foundation.
*Address correspondence to this author.
‡The Miriam Hospital.
§University of Lowell.

[1] Abbreviations: HDL, high-density lipoproteins; apo, apolipoprotein; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] Animals studied were housed at the New England Primate Regional Research Center, Southboro, MA.

0006-2960/87/0426-1457$01.50/0