J. Biochem. **99**, 1253–1265 (1986)

Structures of Oligomannoside Chains of α-Mannosidase from Porcine Kidney[1]

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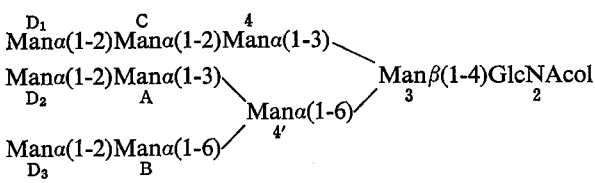
Received for publication, November 25, 1985

Lysosomal acid α-mannosidase from porcine kidney was found to contain mannose (4.8%), galactose (0.9%), fucose (0.5%), *N*-acetylglucosamine (3.1%), and mannose 6-phosphate (0.1%). Approximately 50% of the total hexose of the oligosaccharide chains could be released by endo-β-*N*-acetylglucosaminidase-H (endo-H). They were predominantly neutral, oligomannoside-type oligosaccharides containing 5, 6, and 9 mannose residues, respectively, in the centesimal ratio of 36 : 25 : 34. 500-MHz ^{1}H-NMR spectroscopy in conjunction with sequential exoglycosidase digestion of the reduced compounds revealed that each of the three fractions consisted of a single isomer only; the Man_9 compound has the following structure:



The Man_6-compound lacks Man residues D_1, D_2, and D_3, while the Man_5-compound lacks Man-C as well. In addition to the neutral ones, some (5%) phosphorylated oligomannoside-type oligosaccharides were obtained.

The endo-H resistant glycopeptides were subjected to hydrazinolysis. Approximately 60% of the oligosaccharides released by hydrazine were found to be of rather small size; their composition can be represented as $Man_{2-3}GlcNAc[Fuc]_{0-1}GlcNAcol$.

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture and Science of Japan, by the Netherlands Foundation for Chemical Research (SON/ZWO) and by Grant UUKC-83-13 from the Netherlands Cancer Foundation (KWF).

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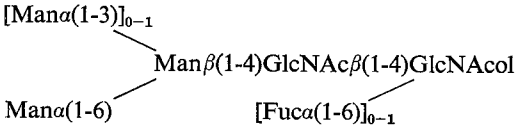
Abbreviations: endo-H, endo-β-*N*-acetylglucosaminidase.

The remaining 40% consist of larger-size, galactose-containing, *N*-acetyllactosamine-type oligosaccharides.

Studies involving sequential exoglycosidase digestion and 500-MHz ^{1}H-NMR spectroscopy performed on the highly purified small-sized compounds revealed the following four structures for the endo-H-resistant oligosaccharides:



Lysosomal hydrolases form a special class of glycoproteins that possess recognition markers for carbohydrate-specific receptors. One of these markers is a phosphorylated oligosaccharide which serves a critical role in mediating the endocytosis and intracellular transport of these enzymes in connective tissue-type cells (reviewed in Ref. *1*). The other is an oligomannoside-type oligosaccharide which is recognized by the clearance system present on both alveolar macrophages and sinusoidal cells of the liver (*2–7*). In addition, the latter is essential for the recognition by a binding protein (mannan-binding protein) specific for oligomannoside-type oligosaccharides, which is located in the endoplasmic reticulum of the liver (*7–13*). In spite of these intriguing observations, insufficient material has precluded detailed analyses of the carbohydrate structures of lysosomal hydrolases, with a few exceptions (*14–17*).

We developed a large scale purification procedure for α-mannosidase, one of the lysosomal hydrolases, from porcine kidney and studied its oligosaccharide structures. α-Mannosidase from porcine kidney binds to the mannan-binding protein with high affinity (*8*, *9*), but is also recognized by the phosphomannosyl receptor on connective tissue-type cells (*18*).

In this paper, we present the results of detailed structural studies on the endo-H-releasable and -resistant oligosaccharides on the α-mannosidase, involving enzymatic analyses with exoglycosidases and high-resolution ^{1}H-NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Materials—Bio-Gel P-2 (minus 400 mesh), AG 50WX4 (100–200 mesh), AG 1X2 (100–200 mesh), and AG 3 (100–200 mesh) were purchased from Bio-Rad Laboratories, Richmond. Sephadex G-25 (fine), Sephadex G-50 (fine), and Sepharose CL-6B were from Pharmacia, Uppsala. NaB[^{3}H]$_4$ (469 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. [1-^{14}C]Acetic anhydride (10.0 mCi/mmol) was from New England Nuclear, Boston. $Man_7GlcNAcol$, $Man_6GlcNAcol$, and $Man_5GlcNAcol$ were prepared from cathepsin D from porcine spleen as described previously (*16*). Manβ-(1→4)GlcNAcol was prepared from $Man_5GlcNAcol$ by exhaustive digestion with jack bean α-mannosidase. Fresh porcine kidneys were obtained from a local slaughterhouse.

Endo-β-*N*-acetylglucosaminidase H (endo-H) from *Streptomyces griseus*, α-L-fucosidase from *Charonia lampas* and jack bean β-*N*-acetylhexosaminidase were obtained from Seikagaku Kogyo Co., Tokyo. Pronase P was purchased from Kaken Kagaku Co., Tokyo. Purified α(1→2)-specific mannosidase from *Aspergillus saitoi* (*19*, *20*) was kindly provided by Dr. Eiji Ichishima, Tokyo Noko University, Tokyo. Jack bean α-mannosidase was prepared according to Li and Li (*21*) and β-mannosidase from a snail, *Achatina fulica*, according to Sugahara and Yamashina (*22*).

Methods—Purification of α-mannosidase from porcine kidney: α-Mannosidase was purified by a modification of the method of Okumura and Yamashina (*23*). All operations were carried out at 4°C unless otherwise stated. Fresh porcine kidneys (5 kg) were homogenized in a Waring blendor with 20 liters of 1 mM phosphate buffer, pH 7.0, for 10 min. The homogenate was left standing for 1 h and then centrifuged at 10,000 rpm for 20 min. Throughout the following isolation procedures, 1 mM phosphate was included in all solutions and buffers to protect the α-mannosidase from attack by tissue phosphatases. The

supernatant was brought to 40% saturation with respect to ammonium sulfate, and then centrifuged at 10,000 rpm for 20 min. To the supernatant, solid ammonium sulfate was added to 70% saturation, and the resulting precipitate was collected by centrifugation. The sediment was dissolved in 1 mM phosphate buffer, pH 7.0, followed by dialysis. The dialysate was made to 1% protein, and then ammonium sulfate and Zn acetate were added to 0.12 M and 0.01 M, respectively. The solution was adjusted to pH 6.0 with 1 M acetic acid, and then cold 50% acetone, made up of equal volumes of acetone and 0.01 M Zn acetate buffer, pH 5.9, was added to the solution with stirring to give an acetone concentration of 15%. After centrifugation, the supernatant was brought to 35% acetone concentration by further addition of the 50% acetone solution. The resulting precipitate was collected by centrifugation and then dissolved in 0.02 M glycine–NaOH buffer, pH 10.5. The solution was made to 0.12 M ammonium sulfate and then adjusted to pH 6.6 with 1 M acetic acid. The precipitate formed was removed by centrifugation and the supernatant was heated at 60°C for 15 min. The resulting slightly turbid solution was clarified by centrifugation. To the supernatant, ammonium sulfate was added to 80% saturation and the resulting precipitate was collected by centrifugation. The sediment was dissolved in 0.05 M phosphate buffer, pH 6.8, and the solution was dialyzed against the same buffer. The dialysate was applied to a column of hydroxyapatite (5 × 10 cm) equilibrated with the same buffer. Elution was carried out in a stepwise manner with 0.12 M and 0.2 M phosphate buffer, pH 6.8. The fractions containing α-mannosidase activity eluted with 0.2 M phosphate buffer were combined, concentrated, and then applied to a Sepharose CL-6B column (1.8 × 185 cm) equilibrated with 0.05 M phosphate buffer, pH 6.8. Fractions containing α-mannosidase activity were combined, concentrated, and then rechromatographed on the same Sepharose column. Through these procedures, approximately 50 mg protein of the purified α-mannosidase with a specific activity of 15.4 units per mg protein was obtained from 5 kg porcine kidneys with a 21% recovery from the homogenate and a 2,170-fold purification.

Enzymatic digestions: Digestions with Pronase P, endo-H, jack bean α-mannosidase, *Aspergillus saitoi* α-mannosidase, and snail β-mannosidase were performed as described previously (*16*). Digestions with β-*N*-acetylhexosaminidase and α-L-fucosidase were performed according to Yamashita *et al.* (*24*).

Hydrazinolysis and Smith degradation: Hydrazinolysis and Smith degradation were performed according to Takasaki *et al.* (*25*) and Yoshima *et al.* (*26*), respectively.

Paper chromatography: Descending paper chromatography was performed on borate-impregnated Toyo No. 51A paper with Solvent I, ethylacetate/isopropanol/pyridine/water (7 : 3 : 2 : 2, v/v) (*27*), and Toyo No. 51A paper with Solvent II, ethylacetate/pyridine/acetic acid/water (5 : 5 : 1 : 3, v/v).

Gel permeation chromatography: Bio-Gel P-2 column chromatography was performed at 55°C on a column equipped with a water jacket. For analytical purposes, the column (1.8 × 190 cm) was eluted with water, and for preparative purposes, it (1.5 × 190 cm) was equilibrated and then eluted with 50 mM pyridine-acetic acid buffer, pH 5.0. Calibration of the column was carried out with glucose oligomers and oligosaccharides of known structure, *e.g.* Man_{5-7}GlcNAcol and Manβ(1→4)-GlcNAcol (*28*).

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was performed with system A at pH 9.45 as described by Rodbard and Chrambach (*29*).

Analytical methods: Hexose was determined by the orcinol-H_2SO_4 method of Hewitt (*30*). Sialic acid was determined by the resorcinol method of Jourdian *et al.* (*31*). Hexosamine was determined with a Hitachi amino acid analyzer 835, after hydrolysis in 6 M HCl at 100°C for 16 h. Neutral sugars were separated and estimated by mass-fragmentography after conversion into alditol acetates as described previously (*32*). Mannose 6-phosphate was estimated enzymatically as described previously (*16*). Radioactivity was measured with a Beckman liquid scintillation spectrophotometer, model LS 7500, with a toluene–Triton X-100 scintillation mixture (*33*).

500-MHz ^{1}H-NMR spectroscopy: The conditions for 500-MHz ^{1}H-NMR spectroscopy were as described in a previous paper (*16*). The spectra were recorded at various probe temperatures (27, 40, 52°C) in order to visualize the complete

anomeric-proton regions. The chemical shifts listed in Tables II and III (and mentioned in the text), however, were measured at 27°C, unless otherwise stated.

RESULTS

Carbohydrate Composition of α-Mannosidase—Upon polyacrylamide gel electrophoresis at pH 9.45, the purified sample was found to be homogeneous (data not shown). The enzyme contained mannose (4.8%), galactose (0.9%), fucose (0.5%), glucose (0.1%), *N*-acetylglucosamine (3.1%), and mannose 6-phosphate (0.1%); these values, when expressed mol per mol of the enzyme, corresponded to 26, 5.0, 2.8, 0.6, 14, and 0.3, respectively. The high content of mannose suggested that oligomannoside-type oligosaccharides are dominant in the carbohydrate moiety of the enzyme. They seemed to be partly phosphorylated, as judged from the value for mannose 6-phosphate (0.1% on a weight basis) which corresponded to 0.3 mol per mol of the enzyme. The occurrence of galactose in a significant amount suggested that the enzyme also contains *N*-acetyllactosamine-type oligosaccharides.

Isolation of Oligosaccharides by Endo-H Digestion—Purified α-mannosidase (49 mg protein) was exhaustively digested with Pronase P under the conditions with which the activities of phosphatase and phosphodiesterase detectable in Pronase P were completely inhibited (*16*). The resulting glycopeptides were isolated by successive gel filtrations on columns of Sephadex G-50 (1.2 × 120 cm) and Sephadex G-25 (1.5 × 110 cm). Fractions positive for the orcinol-H_2SO_4 reaction were collected.

The NH_2-termini of the isolated glycopeptides were then labeled with [1-^{14}C]acetic anhydride as described previously (*16*) to facilitate monitoring of the peptide moieties of the glycopeptides in the following experiments. The labeled glycopeptides were digested with 0.2 units of endo-H in 200 μl of 50 mM citrate-phosphate buffer, pH 5.0, at 37°C for 12 h with a few drops of toluene. Subsequently, another 0.1 unit of the enzyme was added. The digestion was continued for another 12 h, until the reducing power of the reaction mixture due to the released oligosaccharides, as monitored by the method of Park and Johnson (*34*), had reached a plateau. The digest was heated in boiling water for 2 min to inactivate the enzyme, and was then applied to a Sephadex G-25 column (1.5 × 110 cm). Fractions positive for the orcinol-H_2SO_4 reaction were collected. One-fifth of the pooled sample was reduced with 1 mCi of $NaB[^3H]_4$ under the conditions described previously (*16*), and the rest with an excess of $NaBH_4$ under the same conditions. The reduced oligosaccharides and

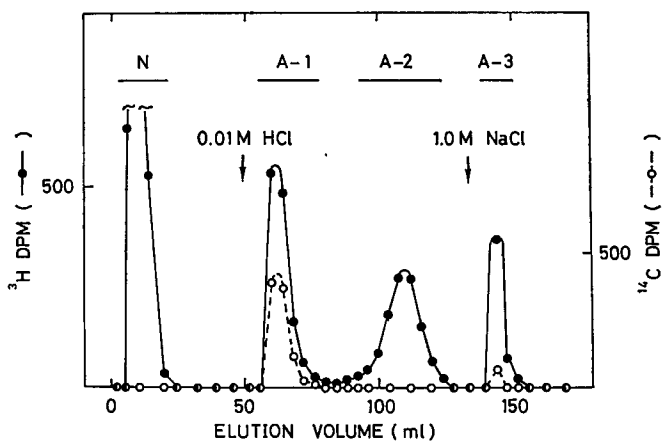


Fig. 1. AG 1X2 chromatography of the endo-H digest. The endo-H digest was applied to a AG 1X2 (Cl^- form) column (0.9 × 17 cm) after reduction with $NaB[^3H_4]$. Elution was carried ou in a stepwise manner with 0.01 M HCl and 1.0 M NaCl, as indicated by arrows. Fractions of 2.0 ml were collected and aliquots thereof were used for monitoring radioactivity.

undigested glycopeptides were purified by gel filtration on a Sephadex G-25 column (1.5 × 110 cm), followed by passage over a small AG 50WX4 column (H^+ form). Subsequently, they were fractionated by chromatography on an AG 1X2 (Cl^- form) column (0.9 × 17 cm). Elution was carried out in a stepwise manner with 0.01 M HCl and 1 M NaCl. As shown in Fig. 1, ^{3}H-radioactivities were separated into four fractions, *i.e.* N, A-1, A-2, and A-3. Of these fractions, N, A-1, and A-2 were positive for the orcinol-H_2SO_4 reaction, containing 46%, 49%, and 5% of the total hexose, respectively. To detect ^{3}H-*N*-acetylglucosaminitol, which should have originated from the reducing termini of the released oligosaccharides, an aliquot of each of these fractions was hydrolyzed in 4 M HCl at 100°C for 4 h, and then the hydrolysates were analyzed after re-*N*-acetylation by paper chromatography on borate-impregnated Toyo No. 51A paper with Solvent I. Only N and A-2 produced ^{3}H-*N*-acetylglucosaminitol, neither A-1 nor A-3 doing so. The ^{3}H-radioactivities in A-1 and A-3 seemed to be due to contaminating peptides (unidentified) rather than to released oligosaccharides. Thus, N and A-2 were designated as endo-H-releasable oligosaccharides and the carbohydrate moiety of A-1 as endo-H-resistant oligosaccharides. N and A-1 were subjected to the following structural analyses. The results of detailed analyses of A-2 which include H-NMR data for phosphorylated oligosaccharides will be reported elsewhere. A-3 was not investigated further.

Fractionation and Characterization of Fraction N—Fraction N, comprising 90% of the endo-H-releasable oligosaccharides, was fractionated on a Bio-Gel P-2 column to give three major subfractions, N-1, N-2, and N-3, which accounted for 36%, 25%, and 34%, respectively, of the total ^{3}H-radioactivity (Fig. 2A). N-1, N-2, and N-3 were eluted at positions corresponding to Man_9-GlcNAcol, Man_6GlcNAcol and Man_5GlcNAcol, respectively.

Fraction N was further characterized by means of digestions with various glycosidases. Treatment of fraction N with *A. saitoi* α(1→2)-specific mannosidase gave a single peak on gel filtration at the elution position of N-3 (Fig. 2B), indicating that N-1 and N-2 comprised four and one additional α(1→2)-linked mannose residues at the nonreducing termini of N-3, respectively. Digestion of the oligosaccharides in this peak with jack bean α-amnnosidase produced a single component that was eluted at the position of ManGlcNAcol, indicating that four α-mannose residues were attached to the ManGlcNAcol core (Fig. 2C). Most of the jack bean α-mannosidase digests were, in turn, converted to GlcNAcol on digestion with β-mannosidase (Fig. 2D). Based on these lines of evidence and taking the specificity of endo-H (*35–37*) into account, the structures of N-1, N-2, and N-3 were deduced to be (Manα1→2)$_n$αMan$_4$-

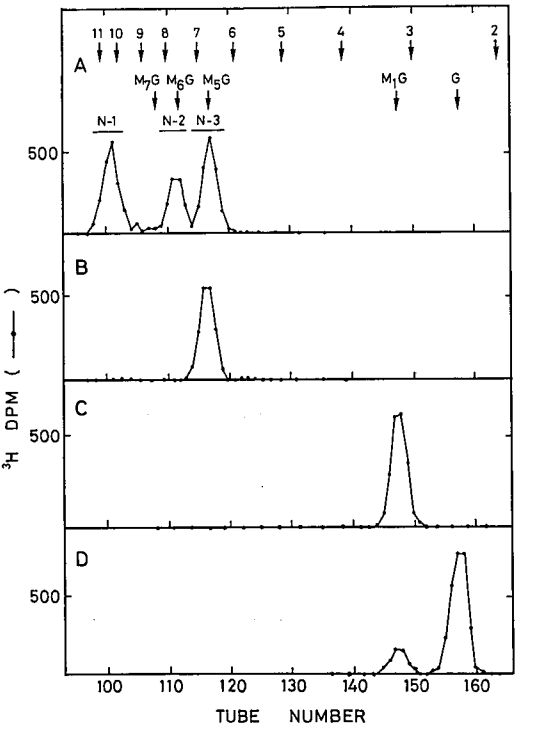


Fig. 2. Gel permeation chromatography of the neutral oligosaccharides and their enzymatic digests. The radioactive oligosaccharides were applied to a Bio-Gel P-2 column (1.8 × 190 cm) which was then developed with water. Fractions of 1.9 ml were collected and aliquots thereof were used for monitoring radioactivity. A, the neutral oligosaccharides (Fig. 1, N) (117,000 dpm); B, α1→2-specific mannosidase digest of A (55,000 dpm); C, jack bean α-mannosidase digest of B (25,000 dpm); D, β-mannosidase digest of C (10,000 dpm). The arrows denote the elution positions of glucose oligomers, with numbers indicating the numbers of glucose units and authentic samples (M_7G, Man_7GlcNAcol; M_6G, Man_6GlcNAcol; M_5G, Man_5-GlcNAcol; M_1G, ManGlcNAcol; G, GlcNAcol).

βManGlcNAcol (n=4, 1, and 0 for N-1, N-2, and N-3, respectively). Further characterization of each fraction was carried out by means of 500 MHz ^{1}H-NMR spectroscopy.

The chemical shifts of the structural-reporter groups of N-1 to N-3 are listed in Table I. The 500-MHz ^{1}H-NMR spectrum of N-1 shown in Fig. 3, as a typical example, shows the involvement of a $Man_9GlcNAcol$ compound. Comparison of the NMR-data for N-1 with those for $Man_9GlcNAc_2$-Asn (compound 72 in Ref. *38*) and $Man_9GlcNAc$-GlcNAcol (F1 in Ref. *39*) revealed that all three structures are the same with respect to the arrangement of the Man-residues. GlcNAc2-ol is characterized by the presence of a singlet in the *N*-acetyl region of the spectrum (δ 2.059) and by its characteristically-shaped H-2 resonance at δ 4.217 (compare with in Ref. *16*). Therefore, the structure of N-1 is

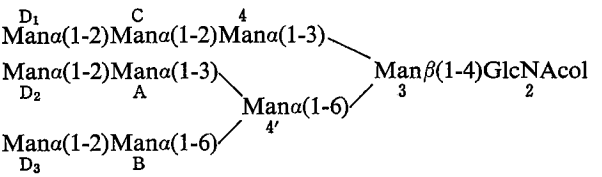


TABLE I. ^{1}H chemical shifts of structural-reporter groups of constituent monosaccharides of the endo-H releasable oligosaccharides obtained from porcine-kidney α-mannosidase.

Reporter group	Residue	Chemical shift[a] in[b] N-1	N-2	N-3
		D_1-C-4 D_2-A 3-2ol 4' D_3-B	C-4 A 3-2ol 4' B	4 A 3-2ol 4' B
H-1	3	4.800[c]	4.801[c]	4.811[c]
	4	5.331	5.341	5.097
	4'	4.872	4.883	4.880
	A	5.392	5.136	5.135
	B	5.142	4.907	4.905
	C	5.306	5.055	—
	D_1	5.046	—	—
	D_2	5.046	—	—
	D_3	5.037	—	—
H-2	2ol	4.217	4.218	4.216
	3	4.236	4.240	4.256
	4	4.110	4.119	4.079
	4'	4.171	4.179	4.178
	A	4.094	4.063	4.061
	B	4.024	3.993	3.984
	C	4.101	4.071	—
	D_1	4.073	—	—
	D_2	4.073	—	—
	D_3	4.061	—	—
NAc	2ol	2.059	2.058	2.059

[a] Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) but were actually measured relative to internal acetone (δ 2.225), in D_2O at 27°C. [b] For the complete structures of the compounds, see Table III. [c] Value determined at 40°C.

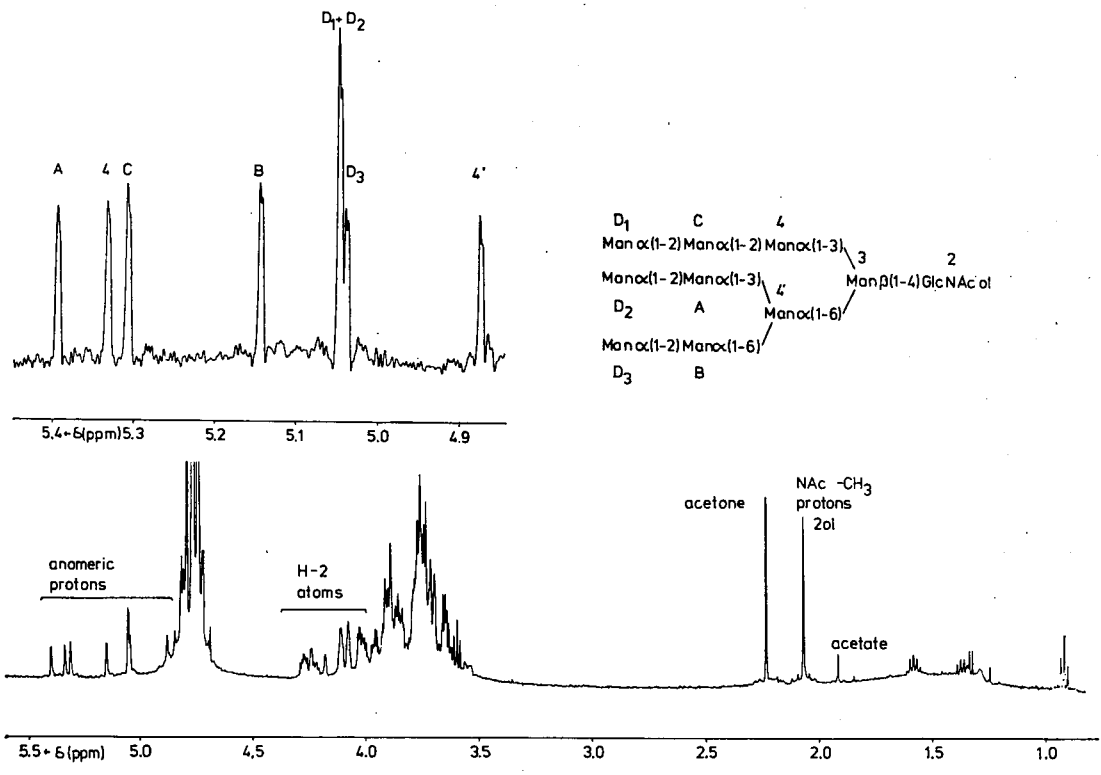
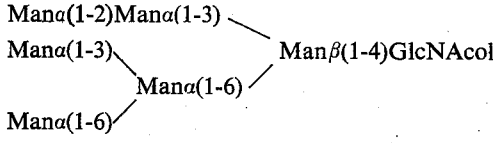


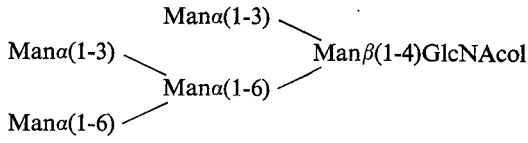
Fig. 3. 500-MH ^{1}H-NMR spectrum (D_2O, 27°C) of neutral oligosaccharide-alditol N-1, released from porcine-kidney α-mannosidase by endo-H. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

The chemical shifts of the structural-reporter groups of most of the Man-residues in the Man_9-structure are only moderately influenced by the modification of the authentic *N*-acetylchitobiose unit into GlcNAc2-ol. Only for H-2 of Man-4′, and for H-1 of Man-A and Man-D_2, are the shift effects introduced greater than 0.01 ppm (compare with in Ref. *38*).

The structures of N-2 and N-3 from α-mannosidase are identical to those of N-2b and N-3 from cathepsin D (*16*). This was confirmed the close resemblance with the NMR-data for the corresponding compounds. Therefore, the structure of N-2 is



and that of N-3 is



In line with earlier observations (*39*), the shift effects of reduction of GlcNAc-2 to GlcNAcol on the structural reporters of Man-residues in these smaller compounds are generally greater than those of the conversion of GlcNAc-1 to GlcNAcol (compare with F2 and F3 in Ref. *39*).

Isolation of Endo-H-Resistant Oligosaccharides by Hydrazinolysis—Fraction A-1, which consisted of endo-H-resistant oligosaccharides, was desalted on Sephadex G-25 (1.5 × 110 cm) and then evaporated to dryness. The dried material was suspended in 1.0 ml of anhydrous hydrazine and then subjected to hydrazinolysis followed by re-*N*-acetylation according to Takasaki *et al.* (*25*). The mixture of oligosaccharides released was purified

by gel filtration on a Sephadex G-25 column (1.5 × 110 cm), and fractions positive for the orcinol-H_2SO_4 reaction were collected. Through this purification, all the ^3H- and ^{14}C-radioactivities incorporated into the peptide portion of fraction A-1 were successfully separated from the oligosaccharides. The resulting oligosaccharides were reduced with $NaB[^3H]_4$ and then purified under the same conditions as used for endo-H-releasable oligosaccharides.

Fractionation and Characterization of Endo-H-Resistant Oligosaccharides—Endo-H-resistant oligosaccharides released on hydrazinolysis were fractionated on a Bio-Gel P-2 column, and then pooled into four fractions, F-1, F-2, F-3, and F-4 (Fig. 4). Fraction F-1 seemed to consist of *N*-acetyllactosamine-type oligosaccharides based on the results of sugar composition analyses, and their detailed structures will be reported elsewhere. Fractions F-2, F-3, and F-4 were further fractionated by paper chromatography on Toyo No. 51A paper with Solvent II. As shown in Fig. 5, F-3 was separated into four subfractions, *i.e.* F-3-1, F-3-2, F-3-3, and F-3-4, and F-4 into two, *i.e.* F-4-1 and F-4-2, while F-2 consisted of a single component.

The purified fractions were subjected to sequential exoglycosidase digestion, and the reaction mixtures at each step were analyzed by gel filtration on a Bio-Gel P-2 column. Treatment of F-2 and F-3-4 with jack bean α-mannosidase shifted their elution positions by two and one glucose units, respectively, giving the same single peak at the position of 6.5 glucose units, indicating that two and one α-mannose residues had been removed from the oligosaccharides, respectively. One β-mannose residue was, in turn, liberated from this digest on incubation with β-mannosidase. The product gave 1 mol each of β-*N*-acetylglucosamine and α-L-fucose on further digestion with β-*N*-acetylhexosaminidase and β-L-fucosidase, respectively, and the digest with the two glucosidases comigrated with *N*-acetylglucosaminitol. The structures of F-2 and F-3-4 could therefore be deduced to be $\alpha Man_n\beta ManGlcNAc(\alpha Fuc)GlcNAcol$ ($n=2$ and 1 for F-2 and F-3-4, respectively). The structures of F-3-3 and F-4-2 were similarly

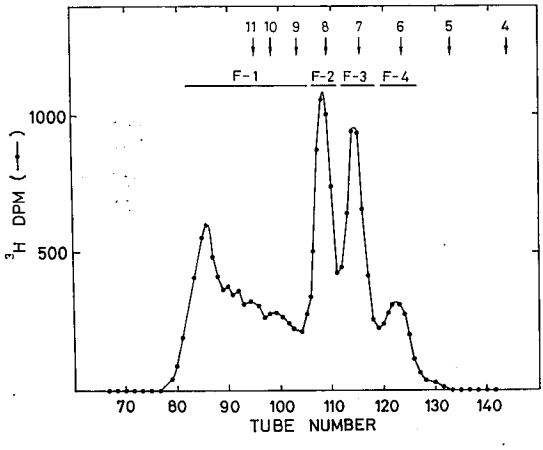


Fig. 4. Gel permeation chromatography of the endo-H-resistant oligosaccharides. The conditions for analysis of radioactive sugars are the same as in Fig. 2.

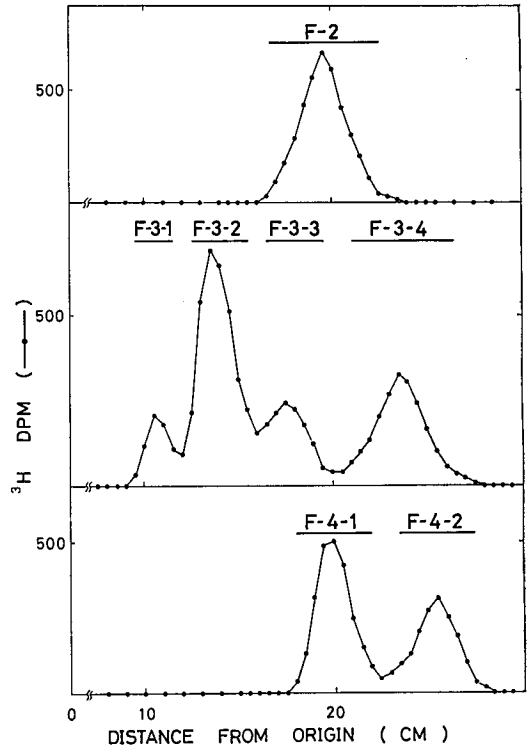


Fig. 5. Paper chromatography of F-2, F-3, and F-4. The radioactive oligosaccharides were subjected to descending paper chromatography with Solvent II for 6 days. After chromatography, the paper was cut into 0.5 cm wide pieces, and the oligosaccharides were extracted with 0.05 M pyridine–acetic acid buffer, pH 5.0. Aliquots of the extracts were used for monitoring radioactivity. A, F-2 (500,000 dpm); B, F-3 (500,000 dpm); C, F-4 (200,000 dpm).

identified as $\alpha Man_n\beta ManGlcNAcGlcNAcol$ ($n=2$ and 1 for F-3-3 and F-4-2, respectively). None of the termini of F-3-1, F-3-2, and F-4-1 were identified as *N*-acetylglucosaminitol, whereas the anomeric configurations and sequences of the monosaccharides, as revealed on exoglycosidase digestion (data not shown), were the same as those for F-3-3 except for the reducing terminal. These three fractions appeared to have modified reducing termini that were produced during the hydrazinolysis. In fact the hexose content values *versus* ^{3}H-radioactivities of these fractions were much lower than that of F-3-3.

Further characterization of F-2, F-3-3, and F-3-4 was carried out by 500-MHz ^{1}H-NMR spectroscopy, while F-4-2 was subjected to Smith degradation.

The relevant 500-MHz ^{1}H-NMR data for compounds F-2, F-3-3, and F-3-4 are listed in Table II. The 500-MHz ^{1}H-NMR spectrum of F-2, as a typical example of the endo-H resistant oligosaccharides, is shown in Fig. 6.

Comparison of the NMR-characteristics of F-3-3 with those of $Man_3GlcNAcGlcNAcol$ obtained from hen ovomucoid (*40*) revealed that the compounds have identical structures. The sets of chemical shifts of H-1 and H-2 of Man-4 (δ 5.106 and 4.063) and Man-4′ (δ 4.916 and 3.973) are typical for the terminal nonreducing positions of these residues (*16*, *38*, *40*). The reduced *N*-acetylchitobiose unit is characterized by the set of *N*-acetyl signals at δ 2.057 and δ 2.077, the GlcNAcol H-2 signal at δ 4.242 and the H-1 doublet of GlcNAc-2 at δ 4.641. (*cf.* Refs. *39*, *40*).

Compound F-2 appears to be an extension of F-3-3, with a Fuc residue $\alpha(1\rightarrow 6)$ linked to GlcNAcol. This was deduced as follows. The $\alpha(1\rightarrow 6)$ type of linkage of Fuc to GlcNAcol is evident from the set of chemical shifts of the structural-reporter groups of Fuc itself (δ H-1

TABLE II. ^{1}H chemical shifts of structural-reporter groups of constituent monosaccharides of the small-sized endo-H resistant oligosaccharides obtained from porcine-kidney α-mannosidase on hydrazinolysis.

Reporter group	Residue	Chemical shift[a] in[b]		
		F-2	F-3-3	F-3-4
		4 \ 3–2–1ol / 4′ F	4 \ 3–2–1ol / 4′	3–2–1ol / 4′ F
H-1	2	4.706	4.641	4.701
	3	4.788	4.787[c]	4.78
	4	5.104	5.106	—
	4′	4.915	4.916	4.914
	Fuc	4.895	—	4.895
H-2	1ol	4.224	4.242	4.226
	3	4.258	4.253	4.085
	4	4.065	4.063	—
	4′	3.969	3.973	3.967
H-5	Fuc	4.075	—	4.072
CH_3	Fuc	1.225	—	1.225
NAc	1ol	2.056	2.057	2.057
	2	2.085	2.077	2.088

[a] Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) but were actually measured relative to internal acetone (δ 2.225), in D_2O at 27°C. [b] For the complete structures of the compounds, see Table III. [c] Value determined after suppression of the HOD-signal by a WEFT pulse sequence.

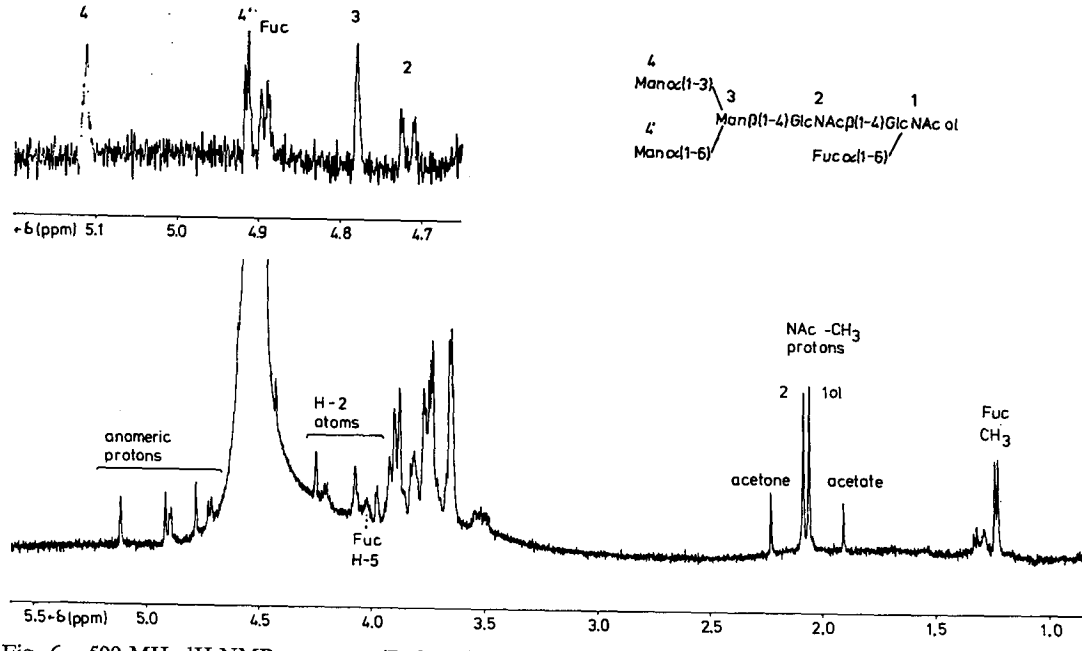
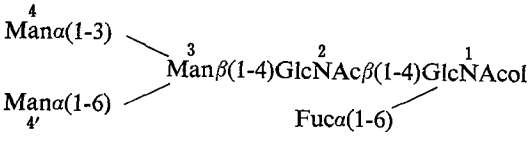
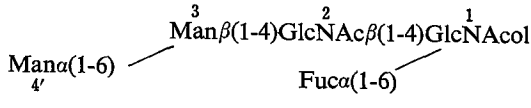


Fig. 6. 500-MHz ^{1}H-NMR spectrum (D_2O, 52°C) of endo-H resistant, hydrazine-released oligosaccharide-alditol F-2, derived from porcine-kidney α-mannosidase. The numbers in the spectrum refer to the corresponding residues in the structure.

4.895, δ H-5 4.075, and δ CH_3 1.225); the latter are in accord with those found for a biantennary, *N*-acetyllactosamine-type oligosaccharide ending in Manβ(1→4)GlcNAcβ(1→4)[Fucα(1→6)]GlcNAcol derived from IgM(ZAJ) (*41*). The effects on the chemical shifts of neighbouring structural-reporter groups, upon the apparent attachment of Fuc to GlcNAcol, are restricted to the H-1 and *N*-acetyl protons of GlcNAc-2 and H-2 of GlcNAcl-ol (see Table II, the step from F-3-3 to F-2). As far as the effects on the GlcNAc-2 reporter groups are concerned, their magnitudes are in line with those observed for α(1→6)-fucosylation of *N*-glycosidic glycopeptides at the Asn-linked GlcNAc (*38*). Therefore, the structure of F-2 is



Fraction F-3-4 contains a $Man_2GlcNAc[Fuc]$-GlcNAcol compound. Comparison of the ^{1}H-NMR chemical shifts of the structural-reporter groups of F-3-4 with those of F-2 (Table II) and those of $Man_2GlcNAc[Fuc]GlcNAcAsn$ (*38*) revealed that in F-3-4 the Man-4 residue is missing, whereas Fuc is again α(1→6)-linked to GlcNAcol. Therefore, the structure of F-3-4 is



In order to determine independently the position of the α-mannose residue in F-4-2, this fraction was subjected to Smith degradation. F-3-3 was used as a reference oligosaccharide. The product from F-4-2 was eluted from a Bio-Gel P-2 column later than the product from F-3-3, Manβ(1→4)GlcNAcβ(1→4)XylNAcol; it differed from the latter by one hexose unit and was eluted at a position corresponding to GlcNAcβ(1→4)XylNAcol. Thus, the structure of F-4-2 was deduced to be Manα(1→6)Manβ(1→4)GlcNAcβ1→4GlcNAcol.

The structures of the neutral oligosaccharides elucidated in this study are summarized in Table III, together with their relative abundances, expressed as moles of oligosaccharide per mol of α-mannosidase estimated on the basis of ^{3}H-radioactivity recovered in each oligosaccharide.

DISCUSSION

The carbohydrate composition of the purified porcine-kidney α-mannosidase shows some remarkable features, *i.e.* a relatively high mannose content, as in other lysosomal enzymes such as cathepsin D and β-glucuronidase (*16*, *42*), but, in addition, the occurrence of galactose and fucose. As will be reported elsewhere, this does not result from the presence of two types of α-mannosidase molecules, a high mannose-type and a complex-type. Rather, the carbohydrate moiety of the enzyme appears to consist of oligomannoside-type oligosaccharides, the major component, and *N*-acetyllactosamine-type oligosaccharides. We were primarily concerned with determining the structures of oligosaccharides releasable on endo-H digestion which should consist of oligomannoside-type oligosaccharides of appropriate size. Endo-H was capable of releasing about 50% of the total hexose. The structures of the neutral oligosaccharides of the endo-H-releasable oligosaccharides were elucidated ultimately by 500-MHz ^{1}H-NMR spectroscopic analysis, and they were found to be of the oligomannoside type (Table III). They were somewhat different in structure from the oligomannoside-type oligosaccharides obtained from

TABLE III. Molar distribution of oligomannoside chains of α-mannosidase.

Fraction	Structure[a]	Distribution[b]
N-1	D_1 Man $\xrightarrow{\alpha1,2}$ C Man $\xrightarrow{\alpha1,2}$ 4 Man $\xrightarrow{\alpha1,3}$ Man$_3$ $\xrightarrow{\beta1,4}$ GlcNAc$_2$ $\xrightarrow{\beta1,4}$ GlcNAc$_1$ $\longrightarrow$ Asn; D_2 Man $\xrightarrow{\alpha1,2}$ A Man $\xrightarrow{\alpha1,3}$ Man $\xrightarrow{\alpha1,6}$ 4′; D_3 Man $\xrightarrow{\alpha1,2}$ B Man $\xrightarrow{\alpha1,6}$ 4′	0.88
N-2	Man $\xrightarrow{\alpha1,2}$ Man $\xrightarrow{\alpha1,3}$ Man$_3$ $\xrightarrow{\beta1,4}$ GlcNAc$_2$ $\xrightarrow{\beta1,4}$ GlcNAc$_1$ $\longrightarrow$ Asn; Man $\xrightarrow{\alpha1,3}$ Man $\xrightarrow{\alpha1,6}$ 4′; Man $\xrightarrow{\alpha1,6}$ 4′	0.61
N-3	Man $\xrightarrow{\alpha1,3}$ Man$_3$ $\xrightarrow{\beta1,4}$ GlcNAc$_2$ $\xrightarrow{\beta1,4}$ GlcNAc$_1$ $\longrightarrow$ Asn; Man $\xrightarrow{\alpha1,3}$ Man $\xrightarrow{\alpha1,6}$ 4′; Man $\xrightarrow{\alpha1,6}$ 4′	0.86
F-2	Man $\xrightarrow{\alpha1,3}$ Man$_3$ $\xrightarrow{\beta1,4}$ GlcNAc$_2$ $\xrightarrow{\beta1,4}$ GlcNAc$_1$ $\longrightarrow$ Asn; Man $\xrightarrow{\alpha1,6}$ 4′; L-Fuc $\xleftarrow{\alpha1,6}$ GlcNAc$_1$	1.44
F-3-3	Man $\xrightarrow{\alpha1,3}$ Man$_3$ $\xrightarrow{\beta1,4}$ GlcNAc$_2$ $\xrightarrow{\beta1,4}$ GlcNAc$_1$ $\longrightarrow$ Asn; Man $\xrightarrow{\alpha1,6}$ 4′	0.57
F-3-4	Man $\xrightarrow{\alpha1,6}$ 4′; Man$_3$ $\xrightarrow{\beta1,4}$ GlcNAc$_2$ $\xrightarrow{\beta1,4}$ GlcNAc$_1$ $\longrightarrow$ Asn; L-Fuc $\xleftarrow{\alpha1,6}$ GlcNAc$_1$	0.54
F-4-2	Man $\xrightarrow{\alpha1,6}$ 4′; Man$_3$ $\xrightarrow{\beta1,4}$ GlcNAc$_2$ $\xrightarrow{\beta1,4}$ GlcNAc$_1$ $\longrightarrow$ Asn	0.26

[a] Structural studies were carried out on oligosaccharides released on hydrazinolysis of the parental structures shown here. [b] Moles of oligosaccharide/mol of α-mannosidase.

cathepsin D (*16*). Unlike in the case of cathepsin D (*16*), for the α-mannosidase, it was not possible to deduce which pathway for the processing of the $Man_9GlcNAc_2$ structure to $Man_5GlcNAc_2$ is dominant due to the absence of $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$.

The endo-H-resistant oligosaccharides were isolated by hydrazinolysis. Approximately 60% were rather short oligosaccharides with only two or three mannose residues. While $Man_2GlcNAc_2$ and $Man_3GlcNAc_2$ are common in the core portions of asparagine linked oligosaccharides, these short oligosaccharides are seldom found in glycoproteins. Recently some other lysosomal enzymes were found to bear $Man_{2-3}GlcNAc[Fuc]_{0-1}GlcNAc$ (*14*, *17*, *43*). Interestingly, the structures detected in these enzymes were single isomers. This might imply that they are the products of processing or degradation from $Man_{5-6}GlcNAc_2$ in an ordered sequence. The metabolic route leading to some smaller-sized structures containing Fuc is not yet understood, and so requires further investigation.

α-Mannosidase from porcine kidney binds strongly to a mannan-binding protein, a hepatic lectin specific for oligomannoside-type oligosaccharides of glycoproteins (*8*, *9*). When assayed as the inhibitory activity towards the binding between the mannan-binding protein and a reference glycoprotein, neoglycoprotein $GlcNAc_{43}$-BSA (bovine serum albumin), the K_I value of the α-mannosidase was determined to be 3.1×10^{-8} M, which is somewhat smaller than that for cathepsin D, 5.4×10^{-6} M (*16*). The higher affinity of the α-mannosidase to the mannan-binding protein may be due to that $Man_9GlcNAc_2$ is dominant in this enzyme whereas this oligosaccharide is absent in cathepsin D.

The results of recent investigations have suggested that there are mechanisms for localizing lysosomal enzymes in lysosomes which are independent of the phosphomannosyl recognition system (*44*, *45*). In view of the high affinity of the lysosomal enzymes to the mannan-binding protein, as discussed above, and the subcellular distribution of the binding protein (*13*), this protein may be involved in the phosphomannose-independent mechanisms.

The ^1H-NMR spectra were recorded at the SON hf-NMR Facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands. The authors wish to thank Miss Hayuru Sakai for her excellent secretarial assistance.

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