Sulfated Asparagine-linked Sugar Chains of Hen Egg Albumin*

(Received for publication, July 11, 1983)

Katsuko Yamashita, Ikuko Ueda, and Akira Kobata†

From the Department of Biochemistry, Kobe University School of Medicine, Chuo-ku, Kobe 650, Japan and the ‡Department of Biochemistry, the Institute of Medical Science, the University of Tokyo, Minato-ku, Tokyo 108, Japan

The fraction of hen egg albumin glycopeptides mixture, which passes through a Dowex 50-H⁺ column, contains two sulfate-containing glycopeptides. Based on the structural studies of oligosaccharides released from the glycopeptides by hydrazinolysis, their structures were elucidated as follows.

Ovalbumin, a major constituent of hen egg white, is a glycoprotein containing 3.5% carbohydrate. Although ovalbumin contains only one asparagine-linked sugar chain in one molecule, an extremely high heterogeneity exists in its sugar moiety as is evidenced by the complicated fractionation pattern of glycopeptides of ovalbumin by Dowex 50-H⁺ column chromatography (1, 2). By the structural study of the oligosaccharides released from the ovalbumin glycopeptides by the action of various endo-β-N-acetylgalosaminidases, structures of nine glycopeptides were elucidated (2–5).

Approximately 4% of the ovalbumin glycopeptides contain acidic oligosaccharides as their carbohydrate moieties and recovered as the pass-through fraction upon Dowex 50-H⁺ column chromatography (2). We have recently studied the structures of these glycopeptides by using hydrazinolysis, a chemical method to cleave specifically GlcNAc→Asn linkage (6). About one-third of the acidic oligosaccharides were converted to neutral oligosaccharides by sialidase digestion. The remaining acidic oligosaccharides were completely resistant to the second sialidase digestion. Structural study of the oligosaccharides in this sialidase-resistant fraction, as will be reported in this paper, revealed that they are two novel sulfate-containing oligosaccharides.

**EXPERIMENTAL PROCEDURES**

**Isolation of Sulfated Oligosaccharides from Hen Egg Albumin—**A mixture of glycopeptides obtained by exhaustive Pronase digestion of crystalline hen egg albumin (34.2 g) was fractionated by Dowex 50-H⁺ column chromatography as described before (2). The fraction (GP-F) which was not retained by the column was collected and subjected to 8 h of hydrazinolysis (6) to release the carbohydrate moieties as oligosaccharides. The oligosaccharide fraction (5 mg, dry weight) was dissolved in 0.5 ml of 0.08 n NaOH. One-tenth of the sample was reduced with NaBH₄ (50 μCi) to obtain a tritium-labeled oligosaccharide fraction, and the remaining sample was reduced with 5 mg of NaBH₄. The yield of the radioactive oligosaccharides was 5.0 × 10⁶ cpm.

When the radioactive sample was subjected to paper electrophoresis at pH 5.4, it was separated into a major and a minor acidic peak with the same mobilities as monosialyl and disialyl biantennary complex-type oligosaccharides, respectively (Fig. 1A). Oligosaccharides in the major radioactive peak were recovered from paper by elution with water and subjected to sialidase digestion. Approximately 30% of the radioactive oligosaccharides were converted to neutral oligosaccharides (Fig. 1B). The oligosaccharides which were resistant to the sialidase treatment were recovered from paper by elution with water. This fraction will be called A-1R in this paper.

*This work has been supported by grants-in-aid for Scientific Research and for Cancer Research, the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

†The abbreviations used are: GlcNAc, N-acetylglucosamine; XylNAc, N-acetyllxosamine. Subscript OT is used in this paper to indicate NaBH₄-reduced sugars. In the same way, subscript OH is used to indicate NaBH₄-reduced sugars. All sugars mentioned in this paper were of D configuration.
vestri et al. (18). The analyses were corrected for inorganic sulfate contamination by assaying unpyrolyzed samples. Desulfation of acidic oligosaccharides was performed by methanolysis reported by Slomi-
any et al. (9). Sulfated oligosaccharides were dissolved in dry methanol containing 0.05 M HCl and kept at room temperature for 4 h. The solution was evaporated to dryness, and the residues were freed from HCl by evaporation with methanol three times. Glycosidic linkages including sialyl linkages are not cleaved by the treatment. However, partial de-N-acetylation from N-acetylglucosaminyl residue is inevitable when the method was applied to oligosaccharides with N-substituted amino or N-acetylglucosaminyl residues as their reducing termini. Therefore, the reaction product was dissolved in saturated NaHCO₃ and N-acetylated with acetic anhydride before paper electrophoretic analysis. Phosphate was analyzed by the method of Chen et al. (10).

Negative fast atom bombardment mass spectrometry was performed by using a JEOL model JMS-DX 300 mass spectrometer (JEOL, Ltd., Tokyo). Agarose gel filtration chromatography, it was separated into two components as shown in previous papers (15-17).

Aspergillus the method of Glasgow et al. (11). α-Mannosidases were purified from Aspergillus saitoi (12) and from jack bean meal (13) according to the cited references. Sialidase purified from Arthrobacter ureafaciens (14) was purchased from Nakarai Chemicals, Ltd., Kyoto. Alkaline phosphatase (Escherichia coli, Type I) was purchased from Sigma.

Other analytical methods and materials were the same as described previously.

Fig. 1. Paper electrophoresis at pH 5.4 of oligosaccharides. Arrows indicate the positions where standard radioactive oligosaccharides migrated: 1, lactitol; 2, NeuAcα2→6Galβ1→4GlcNAcβ1→2Manβ1→6 or 3Galβ1→4GlcNAcβ1→2Manβ1→6 or 3Manβ1→6Galβ1→4GlcNAcβ1→2Manβ1→6( NeuAcα2→6Galβ1→4GlcNAcβ1→2Manβ1→6 or 3Manβ1→4GlcNAcβ1)→4GlcNAcβ1. A, radioactive oligosaccharides liberated from GP-F by hydrazinolysis; B, the radioactive peak A-IR subjected to methanolysis and then N-acetylation; C, radioactive peak A-1R subjected to methanolysis; D, component b subjected to methanolysis and then N-acetylation; E, component b subjected to methanolysis and then N-acetylation.

RESULTS

Characteristics of Oligosaccharides in A-1R Fraction and Their Isolation—Fraction A-1R was completely resistant to sialidase digestion and alkaline phosphatase digestion. However, it was converted to neutral oligosaccharides after methanolysis followed by N-acetylation (Fig. 1C). Since the last result suggested that the oligosaccharides in fraction A-1R may contain sulfate residues as their acidic constituent, analysis for sulfate in A-1R was performed. By use of pyrolysis in combination with a rhodizonate procedure, we found an average of 0.8 mol of sulfate/mol of oligosaccharides calculated on the basis of their radioactivities. No phosphate residue was found.

When the radioactive A-1R was subjected to paper chromatography, it was separated into two components as shown in Fig. 2. The radioactive A-1R fraction. Sample was spotted on Whatman No. 3MM paper and developed with ethyl acetate/pyridine/acetid/water (5:5:1:3) for 93 h. Arrow indicates the position where authentic Manα1→6Manα1→3Manβ1→4GlcNAcβ1→4GlcNAcβ1 migrated.

Fig. 2. Paper chromatography of the radioactive A-1R fraction. Sample was spotted on Whatman No. 3MM paper and developed with ethyl acetate/pyridine/acetid/water (5:5:1:3) for 93 h. Arrow indicates the position where authentic Manα1→6Manα1→3Manβ1→4GlcNAcβ1→4GlcNAcβ1 migrated.

Fig. 3. Bio-Gel P-4 (under 400 mesh) column chromatography of radioactive oligosaccharides. Black arrows indicate the eluting positions of glucose oligomers (numbers indicate the glucose units), and white arrows indicate the migrating positions of standard oligosaccharides: 1, Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manα1→6Manα1→3)Manβ1→6Manα1→3)Manβ1→6Galβ1→4GlcNAcβ1→4GlcNAcβ1; 2, Manα1→6Manα1→3)Manβ1→6Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAcβ1→4GlcNAcβ1→4GlcNAcβ1→4GlcNAcβ1; 3, Manα1→6Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAcβ1→4GlcNAcβ1→4GlcNAcβ1; 4, GlcNAcβ1→4XylNAcβ1 A, the radioactive peak (a-N) in Fig. 1D; B, the radioactive peak (b-N) in Fig. 1E; C, the radioactive peak in A incubated with β-galactosidase (0.5 milliunits/50 pl at 37 °C for 18 h); D, the radioactive peak in B or C incubated with diplococcal β-N-acetylhexosaminidase (6 milliunits/50 pl at 37 °C for 18 h); E, the radioactive peak in D subjected to periodate oxidation; F, the radioactive peak in E subjected to periodate oxidation.
in Fig. 2. They were recovered from paper by elution with water and named components a and b.

Structural Studies of Components a and b—By methanolysis followed by N-acetylation, both components a and b were completely converted to neutral oligosaccharides (Fig. 1, D and E). These neutral oligosaccharides were named a-N and b-N, respectively. Upon Bio-Gel P-4 column chromatography, a-N gave a single radioactive peak with mobility of 12.3 glucose units (Fig. 3A) and b-N also gave a single peak with mobility of 11.1 glucose units (Fig. 3B). The radioactive component in Fig. 3A was converted to a radioactive oligosaccharide with mobility of 11.1 glucose units releasing a galactose residue by β-galactosidase digestion (Fig. 3C). Sequential exoglycosidase digestion of the radioactive components in Fig. 3, B and C, and methylation analysis of their nonradioactive counterparts gave exactly the same results as obtained by the structural study of oligosaccharide C liberated from bovine rhodopsin (17) (data not shown). Therefore, b-N and degalactosyl a-N should have the following structure.

\[
\text{Man}^1 \rightarrow 6 \text{Man}^1 \rightarrow 3 \text{Man}^1 \rightarrow 4 \text{GlcNAc}^1 \rightarrow 4 \text{GlcNAcO}^1 \rightarrow \text{Gal}^1 \rightarrow 4 \text{GlcNAc}^1 \rightarrow 2 \text{Man}^1
\]

These following data support the octasaccharide structure. By diplococcal β-N-acetylhexosaminidase digestion, which specifically cleaves GlcNAcβ1→2Man linkage (27), 1 mol of N-acetylglucosamine was released from the radioactive components in Fig. 3, B and C (Fig. 3D). By two cycles of periodate oxidation, the radioactive component in Fig. 3D was converted to Manα1→6Manβ1→4GlcNAcβ1→4XylNAcO (Fig. 3E) and then to GlcNAcβ1→4XylNAcO (Fig. 3F).

Comparative methylation analysis of components a-N and b-N revealed that a 3,4,6-tri-O-methyl 2-N-methylacamido-2-deoxyglucitol found in b-N was not detected in a-N (Table I). Therefore, component a-N should have the following structure.

\[
\text{Man}^1 \rightarrow 6 \text{Man}^1 \rightarrow 6 \text{Man}^1 \rightarrow 3 \text{Man}^1 \rightarrow 4 \text{GlcNAc}^1 \rightarrow 4 \text{GlcNAcO}^1 \rightarrow \text{Gal}^1 \rightarrow 4 \text{GlcNAc}^1 \rightarrow 2 \text{Man}^1
\]

Components a and b are sulfate derivatives of a-N and b-N, respectively. Fast atom bombardment mass spectrometry of components a and b gave m/z 1680 and 1518, respectively, as negative molecular ions: (M – H)⁻ (Fig. 4, B and A). These results indicated that only one sulfate group is included in both components.

Methylation analysis of components a and b gave 2,3,6-tri-O-methylmannitol which was not found in a-N and b-N (Table I). Therefore, the structures of components a and b should be as shown in Fig. 5.

An attempt to determine by exoglycosidase digestion whether the sulfate residue is linked to a particular α-mannosyl residue or evenly distributed to the 2 α-mannosyl residues was not successful, because both acidic oligosaccharides were extremely resistant to exoglycosidase digestion.

| Partially methylated sugar | Molar ratio* |
|---------------------------|-------------|
| Galactitol                | a           |
| 2,3,6-Tetra-O-methyl      | 1.0         |
| (1,3-di-O-acetyl)         | 1.2         |
| Mannitol                  | a-N         |
| 2,3,6-Tetra-O-methyl      | 1.2         |
| (1,3-di-O-acetyl)         | 1.0         |
| 3,6-Tri-O-methyl          | 1.1         |
| (1,3,5-tri-O-acetyl)      | 1.0         |
| 2,3,6-Tri-O-methyl        | 2.0         |
| (1,4,5-tri-O-acetyl)      | 2.0         |
| 2,4-Di-O-methyl           | b           |
| (1,3,5,6-tetra-O-acetyl)  | 2.0         |
| 2-N-Methylacamido-2      | b-N         |
| 2-deoxyglucitol           | 2.0         |
| 1,3,5,6-Tetra-O-methyl    | 0.7         |
| (4-mon-O-acetyl)          | 0.8         |
| 3,4,6-Tri-O-methyl        | 0.8         |
| (1,3-di-O-acetyl)         | 1.1         |
| 3,6-Di-O-methyl           | 2.2         |
| (1,5-tri-O-acetyl)        | 1.8         |

*Numbers in the table were calculated by taking the italic values as 2.0.

**—, not detected.
possible locations to which sulfate is located. This residue was included without exception in the nonsulfated hybrid-type sugar chains that are different from other nonsulfated hybrid-type sugar chains in two points. The first difference is the absence of the bisecting N-acetylglucosamine residue in the sulfated sugar chains. This residue was included without exception in the nonsulfated hybrid-type sugar chains of hen egg albumin (4,5), classified as hybrid-type sugar chains which were found for the first time in this glycoprotein (4,5), containing sugar chains of hen egg albumin in Fig. 5 can be explained by the action of a-mannosidase I1 cannot remove the a-mannosyl residues from hybrid-type sugar chains of bovine rhodopsin (17). Harpaz and Schachter (18) found that a-mannosidase I1 cannot remove the a-mannosyl residues from hybrid-type sugar chains with the bisecting N-acetylglucosamine residue. Therefore, introduction of this residue to the processing intermediates of asparagine-linked sugar chains results in formation of bisected hybrid-type sugar chains as final products. The structural characteristics of the two sulfated sugar chains in Fig. 5 indicate that addition of a single sulfate residue to the processing intermediate may inhibit the action of a-mannosidase II and also inhibit the addition of the sulfate group to the hybrid-type sugar chain results in formation of bisected hybrid-type sugar chains as final products. The structural characteristics of the two sulfated sugar chains in Fig. 5 indicate that addition of a single sulfate residue to the processing intermediate may inhibit the action of a-mannosidase II and also inhibit the addition of the bisecting N-acetylglucosamine residue. Such control mechanism of the sugar chain maturation by sulfation might be an interesting subject for future enzymatic study.

Another difference is that the Galβ1→4GlcNac group of the sulfated sugar chains is located at C-2 of the mannose residue linked α1→3 to the β-mannosyl residue, while the group in nonsulfated hybrid-type sugar chains of hen egg albumin is located at C-4 of the mannose (5). Possibly, introduction of the sulfite group to the hybrid-type sugar chain may inhibit the formation of the GlcNacβ1→4Manα1 group, which might be a better galactosylation site than the GlcNacβ1→2Manα1 group.

During the past few years, sulfated asparagine-linked sugar chains have been found in various sources such as viral coat protein (20), liver and lung of chick embryo (21), luteopins of many mammals (22,23), human vascular endothelial cells (24), and sea urchin (25). Interestingly, the structures of the sugar chains of viral coat protein, luteopins and hen egg albumin are quite different, and the sulfation seems to occur at various sites of the sugar chains. This is a big contrast from the case of phosphorylated asparagine-linked sugar chains, in which phosphate residues are linked always to the a-mannosyl residue (26).

This evidence may indicate that a variety of sulfating enzymes exist in different tissues and play a vital role in cell differentiation and development.

REFERENCES

1. Cunningham, L. W., Ford, J. D., and Rainey, J. M. (1965) Biochim. Biophys. Acta 101, 233-235
2. Tai, T., Yamashita, K., and Kobata, A. (1977) Biochem. Biophys. Res. Commun. 78, 434-441
3. Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Mura- matsu, T., Iwashita, S., Inoue, Y., and Kobata, A. (1975) J. Biol. Chem. 250, 8569-8575
4. Tai, T., Yamashita, K., Ito, S., and Kobata, A. (1977) J. Biol. Chem. 252, 6687-6694
5. Yamashita, K., Tachibana, Y., and Kobata, A. (1977) J. Biol. Chem. 253, 3862-3869
6. Takasaki, S., Mizuochi, T., and Kobata, A. (1982) Methods Enzymol. 83, 263-295
7. Terho, T. T., and Hartiala, K. (1971) Anal. Biochem. 41, 471-476
8. Silvestri, L. J., Hurst, R. E., Simpson, L., and Settine, J. M. (1982) Anal. Biochem. 123, 303-309
9. Slomiany, A., Kojima, K., Banas-Gruska, Z., and Slomiany, B. L. (1981) Biochim. Biophys. Res. Commun. 100, 778-784
10. Chen, P. S., Toribara, T. Y., Jr., and Warner, H. (1956) Anal. Chem. 28, 1756-1758
11. Glasgow, L. R., Paulson, J. C., and Hill, R. L. (1977) J. Biol. Chem. 252, 8615-8623
12. Ichishima, E., Arai, M., Shigematsu, Y., Kumagai, H., and Su- miya-Tanaka, R. (1981) Biochim. Biophys. Acta 658, 45-53
13. Li, Y.-T., and Li, S.-C. (1972) Methods Enzymol. 28, 702-713
14. Uchida, Y., Tsukada, Y., and Sugimori, Y. (1974) Biochim. Biophys. Acta 350, 425-431
15. Yamashita, K., Liang, C.-J., Funakoshi, S., and Kobata, A. (1981) J. Biol. Chem. 256, 1283-1289
16. Yamashita, K., Kamerling, J. P., and Kobata, A. (1982) J. Biol. Chem. 257, 1289-12914
17. Liang, C.-J., Yamashita, K., Muellenberg, C. G., Shichi, H., and Kobata, A. (1979) J. Biol. Chem. 254, 6414-6418
18. Harpaz, N., and Schachter, H. (1980) J. Biol. Chem. 255, 4891-4892
19. Tabas, I., and Kornfeld, S. (1978) J. Biol. Chem. 253, 7779-7786
20. Prehm, P., Scheid, A., and Choppin, P. W. (1979) J. Biol. Chem. 254, 9689-9677
21. Heifetz, A., Kinsey, W. H., Lennarz, W. J. (1980) J. Biol. Chem. 255, 4328-4344
22. Parsons, T. F., and Pierce, J. G. (1980) J. Biol. Chem. 255, 4545-4554
23. Bedi, N., and Roberts, M. K. (1982) J. Biol. Chem. 257, 13581-13586
24. Heifetz, A., and Lennarz, W. (1979) J. Biol. Chem. 254, 6119-6127
25. Tabas, I., and Kornfeld, S. (1980) J. Biol. Chem. 255, 6636-6639
26. Yamashita, K., Okhura, T., Yoshima, H., and Kobata, A. (1981) Biochem. Biophys. Res. Commun. 100, 226-232
Sulfated asparagine-linked sugar chains of hen egg albumin.
K Yamashita, I Ueda and A Kobata

J. Biol. Chem. 1983, 258:14144-14147.

Access the most updated version of this article at http://www.jbc.org/content/258/23/14144

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/23/14144.full.html#ref-list-1