STRUCTURE OF THE CARBOHYDRATE OF ANTIFREEZE GLYCOPROTEINS FROM AN ANTARCTIC FISH

W. Thomas SHIER

Cell Biology Laboratory, The Salk Institute, Post Office Box 1809, San Diego, California 92112, USA

and

Yuan LIN and Arthur L. DeVRIES

Physiological Research Laboratory, Scripps Institution of Oceanography, University of California, San Diego La Jolla, California 92037, USA

Received 3 April 1975

1. Introduction

Certain Antarctic fish, such as *Trematomus borchgrevinki*, have adapted to life at ambient temperatures well below the freezing point of the sera of temperate marine fishes [1]. The serum of this fish has been shown to contain a series of glycoproteins that depress the freezing point of aqueous solutions about 200–500 times as effectively as NaCl on a molal basis [2]. The effective glycoproteins differ only in molecular weight and are composed of repeating units of a diglycosyltripeptide, Ala–Ala–Thr–O–disaccharide, in which the disaccharide has been shown [3], to consist of a β-galactosyl residue bound to an internal α-*N*-acetyl-galactosaminyl residue (see Structure I, in fig.1).

Studies on the formation of chromogen from antifreeze glycoproteins under alkaline conditions

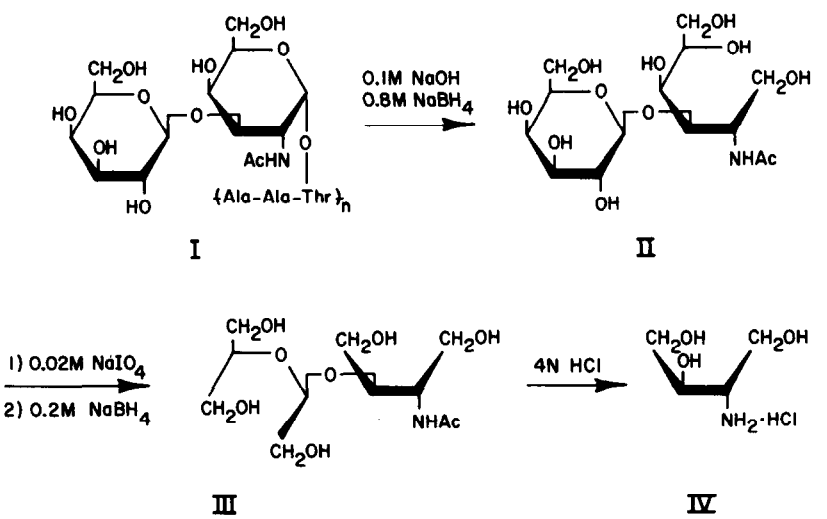

Fig.1. Sequence of reactions employed to degrade a sample of antifreeze glycoproteins from the Antarctic fish *T. borchgrevinki* (I, n=15, 24 and 30) to yield threosaminitol hydrochloride (IV).

have indicated that the glycosidic linkage between the two monosaccharides is 1→3 [4], whereas an analysis of the nuclear magnetic resonance spectral characteristics of the acetylated glycoproteins has indicated that the linkage is 1→4 [5]. These glycoproteins have recently been used to study several types of biological activities lectin binding activity [6], antigenic activity [7] and sialyltransferase acceptor activity [8] in addition to antifreeze activity [5,9]. Since these activities presumably involve the carbohydrate moieties of the glycoproteins, the discrepancy in the reported structures has prompted us to examine the nature of this linkage by a third method involving controlled chemical degradation. The results obtained with this method indicate a 1→3 glycosidic linkage.

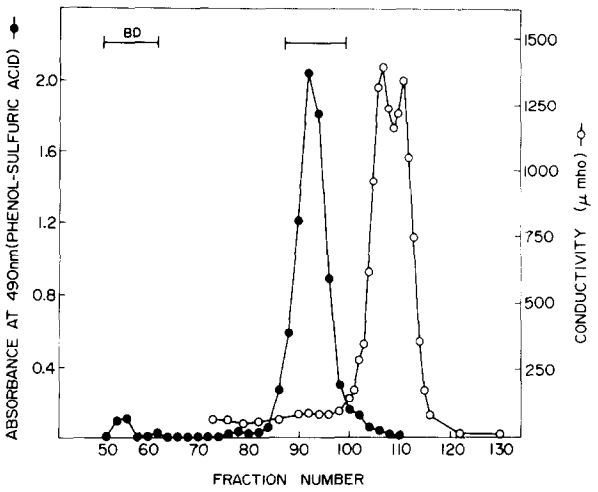

Fig.2. Gel filtration chromatography on Sephadex G-25 of the products of alkaline borohydride degradation of antifreeze glycoproteins after removal of sodium and borate ions. The sample was applied in water to a column (1.2 × 120 cm, equilibrated with water and calibrated with blue dextran (BD)), and eluted with water in 1.2 ml fractions. The diagram shows hexose determinations by the phenol-sulfuric acid method and electrical conductivity measured on a Wheatstone bridge-type conductivity meter. The unlabelled bar over the major hexose peak designates fractions combined for further study; the minor hexose peak in the excluded volume presumably represents carbohydrate not removed from the polypeptide moiety.

2. Materials and methods

L-Threosaminitol and *N*-acetyl-L-arabinosaminitol were the gifts of Dr R. G. Spiro. L-Arabinosaminitol was prepared from *N*-acetyl-L-arabinosaminitol by deacetylation in 4 N HCl for 4 hr at 100°C. D-Galactosaminitol was prepared from *N*-acetyl-D-galactosamine by sodium borohydride reduction and deacetylation according to Spiro and Bhoyroo [10]. D, L-Serinol was purchased from Sigma Chemical Co.

Hexose was determined by the phenol-sulfuric acid method [11]. Conductivity was measured on a Wheatstone bridge-type conductivity meter (Radiometer Copenhagen). Aminopolyols were analyzed on a Beckman Model 121 Amino Acid Analyzer using a 0.9 × 25 cm Beckman PA-35 column eluted with 0.35 M sodium citrate, pH 5.28, at 55°C with a flow rate of 74 ml/hr [12]. Galactosaminitol, arabinosaminitol, threosaminitol and serinol were used as standards, and L-phenylalanine was added to each run as an internal standard.

Antifreeze glycoproteins (100 mg of bands 3–5), prepared and purified as described previously [3], were subjected to alkaline borohydride elimination in 200 ml of 0.8 M $NaBH_4$ in 0.1 M NaOH at 37°C for 68 hr. The bulk of the electrolytes were removed from the reaction mixture by the method of Spiro and Bhoyroo [10], and the remaining reaction products fractionated by gel filtration on Sephadex G-25 as described in the legend to fig.1.

One half of the pooled material in the major hexose peak was oxidized in 41 ml of 0.02 M sodium periodate in 0.05 M sodium acetate buffer, pH 4.5, for 24 hr at 4°C in the dark. The excess sodium periodate was destroyed with 0.5 ml ethylene glycol and the oxidized product reduced without isolation using 144 ml of 0.2 M sodium borohydride in 0.2 M sodium borate buffer, pH 8.0, for 16 hr at 4°C in the dark. The mixture was adjusted to pH 5 with 4 N acetic acid and the sodium and iodate ions removed by passage through coupled columns of Dowex 50-X2, 200–400 mesh (H^+ form, 100 ml) and Dowex 2X8, 200–400 mesh (acetate form, 5 ml). The effluent was evaporated and the boric acid volatilized as methyl borate [10]. The residue containing III was hydrolyzed by heating at 100°C for 4 hr in 4 N hydrochloric acid. The hydrochloric acid was evaporated under vacuum and the non-volatile residue was treated with decolorizing charcoal and lyophilized to yield IV (4.65 mg. 36% of theoretical).

3. Results and discussion

The sequence of reactions outlined in fig.1 converts the *N*-acetyl-galactosamine residue of a serine- or threonine-containing glycopeptide structure to threosaminitol if the *N*-acetylgalactosaminitol derivative, II, contained a 1→3 glycosidic linkage during the periodate oxidation step, but the sequence yields arabinosaminitol if II contained a 1→4 glycosidic linkage [13]. Application of this sequence of reactions to the antifreeze glycoproteins yielded a non-volatile product that contained only threosaminitol (IV) (see fig.3) and small amounts of several impurities of which only arabinosaminitol was identified in trace quantities (less than 2% of threosaminitol present). This result is consistent only with a 1→3 glycosidic linkage, so that the carbohydrate moiety of the antifreeze glycoproteins must have the structure depicted for compound I in fig.1. The assignment of a 1→4 structure to this linkage in antifreeze glycoproteins [5] was based on an interpretation of the nuclear magnetic resonance spectrum of acetylated intact glycoproteins using correlations that were established with monosaccharides and other simple sugars [14]. The results obtained by Vandenheede et al. [4] and those presented above may indicate that spectral correlations established with simple sugars can not be routinely extended to glycoproteins in which case the large size of the molecules can prevent free rotation in solution. Alternatively the anomalous nuclear magnetic resonance spectral characteristics may result from unusual electronic environments caused by either specific intramolecular interactions or glycoprotein-solvent interactions, either of which could be associated with the antifreeze activity of the underivatized glycoproteins [5].

The carbohydrate structure, β-D-galactopyranosyl-(1→3)-α-D-*N*-acetylgalactosaminyl (1→3) threonine or serine, has been observed to be part of carbohydrate moieties from the mammalian glycoproteins fetuin [10] and porcine submaxillary mucin [15]. It may also be part of the *O*-glycosidically linked carbohydrate moieties of IgG and IgA immunoglobulins, erythrocyte membrane protein and chorionic gonadotropin [16]. The observation that antifreeze glycoproteins are acceptors for sialyltransferase enzymes in rat liver suggests that there may also be rat liver glycoproteins that contain this disaccharide as part of larger, sialic acid containing carbohydrate moieties. Consequently, the antifreeze glycoproteins of Antarctic fish provide yet another example of the remarkable degree of conservatism [16] in the structures of the carbohydrate moieties of glycoproteins from a wide range of species.

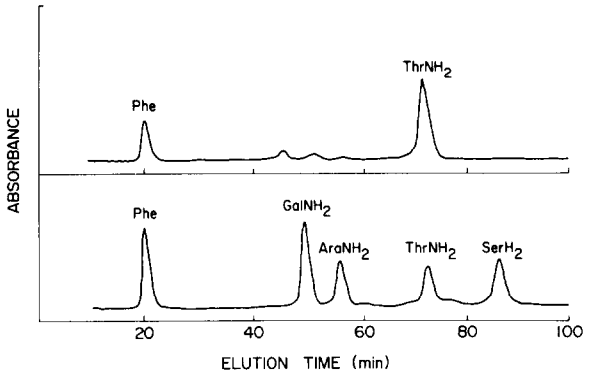

Fig.3. Identification on the amino acid analyzer of threosaminitol resulting from the sequence of reactions outlined in fig.1. Upper panel: hydrolysate of sodium periodate oxidized, sodium borohydride reduced carbohydrate released by alkaline borohydride degradation of antifreeze glycoproteins. Phenylalanine was used as an internal standard. Lower panel: standards; Gal-NH_2, galactosaminitol; Ara-NH_2, arabinosaminitol; Thr-NH_2, threosaminitol; Ser-H_2, serinol.

Acknowledgements

This work was supported by the Theodore Gildred Foundation; U.S. Public Health Service Grants AI 10265 from the National Institute of Allergy and Infectious Diseases, CA 14195 from the National Cancer Institute, GM 21127 from the National Institute for General Medical Sciences; and National Science Foundation grant OPP 74-07917. We thank Ms Cheryl H. Look for performing aminopolyol analyses.

References

[1] De Vries, A. L. and Wohlschlag, D. E. (1969) Science 163, 1073–1075.
[2] De Vries, A. L. (1971) in: Fish Physiology (Hoar, W. H. and Randall, D. J., eds.), Vol. 6, pp. 157–190, Academic Press, New York.

[3] Komatsu, S. K., De Vries, A. L. and Feeney, R. E. (1970) J. Biol. Chem. 245, 2909–2913.
[4] Vandenheede, J. R., Ahmed, A. I. and Feeney, R. E. (1972) J. Biol. Chem. 247, 7885–7889.
[5] Shier, W. T., Lin, Y. and De Vries, A. L. (1972) Biochim. Biophys. Acta 263, 406–413,
[6] Chuba, J. V., Kuhns, W. J., Nigrelli, R. F., Vandenheede, J. R., Osuga, D. T. and Feeney, R. E. (1973) Nature 242, 342–343.
[7] Shier, W. T. (1973) Nature 244, 99–101.
[8] Shier, W. T. and Roloson, G. (1974) Biochem. Biophys. Res. Commun. 59, 51–56.
[9] Ahmed, A. I., Osuga, D. T. and Feeney, R. E. (1973) J. Biol. Chem. 248, 8524–8527.
[10] Spiro, R. G. and Bhoyroo, V. D. (1974) J. Biol. Chem. 249, 5704–5717.
[11] Whistler, R. L. and Wolfrom, M. L. (1962) Methods in Carbohydrate Chemistry, Vol. 1, pp. 388–389, Academic Press, London.
[12] Look, C. H. in preparation.
[13] Spiro, R. G. (1972) Methods Enzymol. 8, 3–43.
[14] Hall, L. D. (1964) Advan. Carbohydr. Chem. 19, 51–93.
[15] Carlson, D. M. (1968) J. Biol. Chem. 243, 616–626.
[16] Spiro, R. G. (1973) Advan. Protein Chem. 27, 349–467.