STUDIES ON THE STRUCTURE AND ACTIVITY OF LOW MOLECULAR WEIGHT GLYCOPROTEINS FROM AN ANTARCTIC FISH*

Yuan Lin+, John G. Duman and Arthur L. DeVries

Scripps Institution of Oceanography

University of California, San Diego

La Jolla, California 92037

Received October 15, 1971

Summary - Two groups of low molecular weight glycoproteins were isolated from the blood serum of an antarctic fish Trematomus borchgrevinki. The complete structures, both the amino acid sequence and the configuration of the carbohydrate moieties, have been studied. The two glycoproteins are similar in structure but different in size. Solutions of these glycoproteins display a thermal hysteresis similar to that of solutions of larger glycoproteins previously isolated from the same fish.

The presence of a group of glycoproteins in the blood sera of antarctic fishes has been reported previously (1, 2). These glycoproteins were isolated and separated according to their electrophoretic mobilities on an acrylamide gel slab into 8 different bands, numbered 1 to 8 proceeding from the cathode to the anode (3). Glycoproteins 1 to 5 vary in size and are larger than 10,500 in molecular weight (3). Since freezing point measurements made with a Fiske Osmometer indicate that these glycoproteins are able to depress the freezing point of water about 200 to 500 times as effectively as NaCl on a molal basis (3, 4), they were termed "active glycoproteins". Recently, it was found that these glycoproteins display an unusual thermal hysteresis; i.e., the freezing points of these glycoprotein solutions differ from the melting points (5).

Glycoproteins 7 and 8 are much smaller than the other glycoproteins, with molecular weights of 3,500 and 2,600, respectively (3). They have similar chemical composition to glycoproteins 1 to 5, except that they contain proline in addition to the alanine and threonine found in the others (3). Previous Fiske Osmometer measurements

* Supported by National Science Foundation Grant GV27327
+ Recipient of National Institutes of Health Postdoctoral Fellowship 1 FO2 HE50405-01

did not reveal that these glycoproteins have unusual freezing point depressing activity; hence they were termed "inactive glycoproteins" (3). Studies on glycoprotein 6 are currently in progress.

In the blood serum of T. borchgrevinki, glycoproteins 7 and 8 occur in a concentration 5 times (on a weight basis) as great as the combined concentration of glycoproteins 1 to 5 (3). The possibility that they might be precursors or reservoirs for the "active glycoproteins" was considered. The structures of glycoproteins 7 and 8 were determined to see whether they have structures consistent with the facile bioconversion of these glycoproteins to active ones. This would be required if they were present as precursor or storage compounds.

Materials and Methods - Glycoproteins 7 and 8 were isolated and purified as described previously (3). Acetylated glycoproteins were prepared according to the method of Komatsu et al. (6). A glycodipeptide fragment was obtained by subtilisin digestion of these glycoproteins according to the method of DeVries et al. (7). α and β-galactosidases were the gifts of Dr. Y. T. Li, Tulane University. All chemicals are of analytical grade; trifluoroacetic acid, pyridine and phenylisothiocyanate were redistilled.

The Edman-dansyl chloride technique (8-10) with polyamide layer chromatography was used to determine the amino acid sequence using 0.15 μmole of each glycoprotein as the starting material. It was not necessary to remove the sugar moiety before sequence determination. The temperature of acid hydrolysis of the dansylated peptide is of critical importance in detecting proline. Under the conditions used (95°C, 16 hours), we were able to detect proline without ambiguity; at an elevated temperature (105°C, 12 hours) no trace of proline can be detected with this technique. All three amino acids show distinct spots on the polyamide layers. After several Edman degradation cycles, traces of the previous amino acids can be detected due to the incomplete cleavages, but intensities of the most recently uncovered amino acids are much greater than the intensities of the products of incomplete cleavage; therefore, the low degree of incomplete degradation does not interfere with the sequence determination.

NMR spectra were obtained on a Jeol JNM-PS 100 instrument. α and β-

galactosidase treatments were carried out according to Li (11). Amino acid analyses were carried out with a Beckman Auto Amino Acid Analyzer (Model 121). Samples were hydrolyzed at 105°C for 20 hours in constant boiling HCl.

Freezing point studies were carried out in a well-controlled temperature-regulating bath (temperature variation, ± 0.01°C) according to the method of Ramsay and Brown (12). The sample solution was placed in a capillary tube which was then sealed and inserted into the bath. The temperature at which an ice crystal began to increase in size was recorded as the freezing point; the temperature at which the last small ice crystal melted was taken as the melting point (5).

Results - The disaccharide moiety found in glycoproteins 3 to 5 has been shown to be a β-D-galactosyl (1→4) α-N-acetylgalactosamine moiety (13). The following lines of evidence indicate that the same carbohydrate moiety occurs in both glycoproteins 7 and 8. (a) Glycopeptides obtained from subtilisin digestion of glycoproteins 7 and 8 were applied to an amino acid analyzer. The chromatogram showed only one single peak in the glycodipeptide region (7) with the same retention time (4.5 hours) as the glycodipeptide obtained from glycoproteins 1 to 5 (7), and this peak did not contain proline. (b) the NMR spectrum of the glycodipeptide (Fig. 1) gave the same pattern of absorptions as did the glycodipeptide from glycoproteins 3 to 5, including absorptions assignable to the anomeric protons H_a and H_b. (c) β-galactosidase, but not α-galactosidase, was able to release galactose from both glycoproteins 7 and 8. (d) The NMR spectrum of acetylated glycoproteins 7 and 8 gave the same pattern of absorptions in the acetyl region as did acetylated glycoproteins 3 to 5.

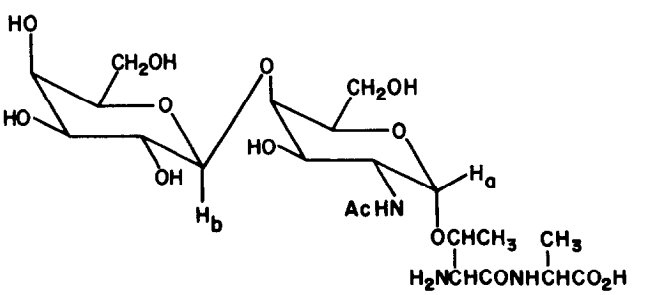
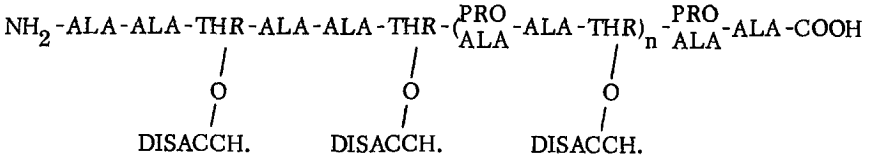


Fig. 1. Structure of glycodipeptide from subtilisin digestion of glycoproteins 7 and 8.



DISACCH. : β-D-galactosyl (1→4) α-N-acetylgalactosamine

Glycoprotein 7, n=4; glycoprotein 8, n=2.

Fig. 2. Complete structures of glycoproteins 7 and 8.

The amino acid sequences of glycoproteins 7 and 8 were determined (Fig. 2) by complete sequential Edman degradation of small samples of each glycoprotein. Glycoprotein 7 contains a polypeptide chain of 20 amino acids; glycoprotein 8, 14 amino acids. Proline residues appear only after the 6th position, following threonine, with concomitant appearance of alanine residues at the same positions. This indicates that glycoproteins 7 and 8 are composed of more than one type of polypeptide chain. Most likely they are groups of glycoproteins containing different numbers of proline residues. In Table I amino acid analysis data on glycoproteins 7 and 8 are compared with data from the Edman-dansyl chloride sequence determinations. The amino acid analysis data yield the ratio of alanine to proline; this ratio could not be determined by the sequencing technique employed in this work. The slightly low values for threonine may have been

Table I

Amino Acid Compositions of Glycoproteins 7 and 8

	Glycoprotein 7		Glycoprotein 8	
Amino Acids	Amino acid analysis data	Sequence determination	Amino acid analysis data	Sequence determination
Threonine	5.3	6	3.8	4
Alanine	11.6 } 14	14	8.4 } 10	10
Proline	2.4		1.6	

caused by the loss of this amino acid during the acid hydrolysis. Molecular weights calculated from the structures determined above give values of 3,800 and 2,600 for glycoproteins 7 and 8. These values are in agreement with those determined by analytical ultracentrifugation (3,500 and 2,600 respectively) (3).

Figure 3 shows the melting points and freezing points of solutions of glycoproteins at various concentrations. Similar thermal hystereses are observed in solutions of all the glycoproteins, but glycoproteins 7 and 8 show a smaller difference between freezing and melting points than do glycoproteins 1 to 5; and they are also not as effective in lowering the temperature of the ice crystal growth. On a weight per volume basis, solutions of glycoprotein 7 have the same freezing points and melting points as do those of glycoprotein 8.

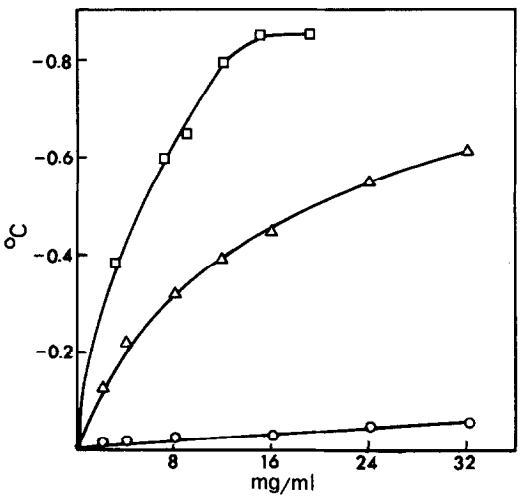


Fig. 3. Freezing points and melting points of aqueous solutions of glycoproteins as a function of concentrations. Freezing points of solutions of glycoproteins 1 to 5, -□-□-; glycoproteins 7 and 8, -Δ-Δ-. The melting points of all the glycoprotein solutions are the same, -o-o-.

Discussion - Since glycoproteins 7 and 8 occur in a relatively high concentration in the blood serum of T. borchgrevinki (3), their physiological significance is of great interest. In previous reports (3,4) they were considered to be inactive in depressing the freezing point of serum, and the possibility was entertained that they might serve as precursors or reservoirs for the "active glycoproteins". It was felt that once the fish encountered a lower environmental temperature, the inactive glycoproteins could be rapidly converted

to active ones. The amino acid sequence data, however, do not seem to be in accord with the foregoing hypothesis, since it would take a very complex enzyme system to remove all the proline residues in various positions in glycoproteins 7 and 8 and to replace them with alanine.

With the Fiske Osmometer we were able to measure a freezing point depressing activity for the larger molecular weight glycoproteins (1 to 5), but not for the smaller ones (7 and 8). However, with the method of Ramsay and Brown (12), we were able to observe a lowering of the temperature of ice propagation even in solutions of glycoproteins 7 and 8. Since the Fiske Osmometer is often calibrated against NaCl solutions, it is evident that care must be taken when interpreting data on non-ideal solutes or physiological solutions. The combined results from the ice crystal growth analysis and the Osmometer measurements may suggest that the smaller glycoproteins have a different mode of action or method of structuring water from that of the glycoproteins of larger molecular weights.

Because of their small sizes, glycoproteins 7 and 8 may be able to carry out some function other than preventing the serum from freezing. For instance, they may be able to travel through cell membranes and prevent intracellular freezing. As mentioned above, the possibility exists that glycoproteins 7 and 8 are really groups of glycoproteins. The members of the groups may be similar in weight but not in function, some possibly serving as precursors of "active" glycoproteins, while others carry out some other physiological function.

References

1. A. L. DeVries, Ph. D. Thesis, Stanford University (1968).
2. A. L. DeVries and D. E. Wohlschlag, Science, 163, 1073 (1969).
3. A. L. DeVries, S. K. Komatsu and R. E. Feeney, J. Biol. Chem., 245, 2901 (1970).
4. A. L. DeVries, in Fish Physiology, W. H. Hoar and D. J. Randall, Eds. Vol. 6, Academic Press, New York, in press.
5. A. L. DeVries, Science, 172, 1152 (1971).
6. S. K. Komatsu, A. L. DeVries and R. E. Feeney, J. Biol. Chem., 245, 2909 (1970).
7. A. L. DeVries, J. Vandenheede and R. E. Feeney, J. Biol. Chem., 246, 305 (1971).
8. W. R. Gray, Methods in Enzymol. 11, 469 (1967).
9. K. R. Woods and K. T. Wong, Biochim. Biophys. Acta, 133, 369 (1967).
10. B. S. Hartley, Biochem. J., 119, 805 (1970).
11. Y. T. Li, Personal communication.
12. J. A. Ramsay and R. H. Brown, J. Sci. Instrum. 32, 372 (1955).
13. W. T. Shier, Y. Lin and A. L. DeVries, submitted for publication.