SIALYLTRANSFERASE ACCEPTOR ACTIVITY OF "ANTIFREEZE" GLYCOPROTEINS FROM AN ANTARCTIC FISH*

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SUMMARY

The "antifreeze" glycoproteins from the Antarctic fish T. borchgrevinki function as effective acceptors for N-acetylneuraminic acid transfered from cytidine 5'-monophosphate N-acetylneuraminic acid by solublized enzymes from rat liver and a rat mammary adenocarcinoma. The properties of the two preparations are sufficiently similar to suggest that the same enzyme(s) are involved. The similarity between the glycoside structure that must result from this reaction and the known glycoside structures of some mucins and blood group substances invites the suggestion that the antifreeze glycoproteins were evolved during adaptation to a freezing environment by loss of the capability of transferring sialic acid to the antifreeze glycoprotein structure.

The "antifreeze" glycoproteins found in the serum of certain Antarctic fish present a striking example of biochemical adaptation to a hostile environment. This unique group of glycoproteins plays an integral role in the mechanisms that permit these fish to resist freezing even at ambient temperatures well below the freezing point of the sera of temperate marine fishes (1). These 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 (2,3) to be β-Gal(1,4 or 1,3)-α-GalNAc. All modifications of the sugar moiety tested to date have destroyed antifreeze activity except conversion of the 6-hydroxymethyl group of galactose to an aldehyde group (2,3). Subsequent conversion of this group to a negatively charged group (carboxylate or bisulfite) resulted in complete destruction of activity. The unique structure and effectiveness of the proteins prompt the

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question of how they evolved, specifically whether they represent a completely new development in evolution or whether they are the result of the adaptation of a preexisting structure to a new use. One approach to answering this question is to investigate other possible metabolic roles of both the native glycoproteins and plausible biosynthetic precursors and products of them.

In this report we describe an additional biological activity of these glycoproteins. They function as efficient acceptors for sialic acid residues transfered from CMP-NANA* by solublized enzymes from two different types of rat tissue, liver and the mammary adenocarcinoma DMBA #8 (4).

MATERIALS AND METHODS

The antifreeze glycoproteins (bands 3-5) were the gift of A. L. DeVries and Y. Lin, prepared as described previously (5). DMBA #8 Fischer rat mammary adenocarcinoma cells were maintained in culture as described previously (4), and tumors were produced by a subcutaneous injection of 1×10^5 cells in the flank of male Fischer F344 rats (Microbiological Associates). Tissue was obtained from tumors less than 25mm in diameter; necrotic tissue was not used.

Particulate enzyme preparations were prepared from liver and tumor tissue by the method of Frot-Coutaz and Got (6). The sialyltransferase activity was solublized by adding 10% Triton X-100 detergent (Rohm and Haas) to give a final concentration of 0.15% Triton X-100 for the liver enzyme and 0.05% for the tumor enzyme. The preparations were stirred at 0° for 30 min and centrifuged at 100,000xg for 1 hr. The pellet was discarded and the supernatant used immediately as the soluble enzyme preparation. Protein was determined by the method of Lowry et al. (7) using bovine serum albumin in the same buffer as standard.

Enzyme activity was determined in reaction mixtures (final volume 0.25 ml) containing 40 mM Tris-citrate buffer at the desired pH (routinely pH 7.0), antifreeze glycoproteins (100 µg), CMP-[^{14}C]-NANA (0.23 nmoles, 0.05 µCi,

* Abbreviations used: NANA, N-acetylneuraminic acid; CMP-NANA, cytidine 5'-monophosphate N-acetylneuraminic acid.

TABLE 1

Comparison of some properties of the NANA transferases solublized from rat liver and tumor tissue

Property	Liver enzyme	Tumor enzyme
Apparent K_m of acceptor (calc. as the conc. of galactose in antifreeze glycoproteins)	0.6 mM	0.6 mM
pH Range of detectable activity (in Tris-citrate)	5.5 - 8.0	5.5 - 8.0
pH Optimum (in Tris-citrate)	7.0	6.5
Mg^{++} or Mn^{++} requirement	None	None

New England Nuclear) and 0.6 mg of enzyme protein. The reaction was initiated by addition of the enzyme preparation, and after incubation for 1 hr at 37°, it was stopped by addition of 0.5 ml of cold 1% phosphotungstic acid in 0.5 N HCl. The precipitate was immediately collected on a Whatman GF/C glass fibre filter, and washed with two 0.5 ml aliquots of cold 1% phosphotungstic acid in 0.5 N HCl. The filter was dried and the [^{14}C] counted in a toluene based scintillator solution.

RESULTS

A membrane bound enzyme preparation from rat liver capable of transferring [^{14}C]-NANA from CMP-[^{14}C]-NANA to endogenous acceptor(s) and to purified antifreeze glycoproteins was solublized with Triton X-100. The incorporation of radioactivity into both endogenous and added acceptor (i. e. radioactivity incorporated above the endogenous activity) was linear over the

TABLE 2

Removal of [^{14}C]-NANA from sialylated antifreeze glycoproteins by Cl. perfringens neuraminidase

Neuraminidase (milliunits)	[^{14}C]-NANA released by neuraminase ± SEM* (cpm)	[^{14}C]-NANA bound after neuraminidase treatment ± SEM* (cpm)
10	388 ± 14	30 ± 7
1	386 ± 14	43 ± 6
10^{-1}	199 ± 52	226 ± 58
10^{-2}	85 ± 56	325 ± 56
10^{-3}	84 ± 23	404 ± 33

* SEM, standard error of the mean for three independent determinations.

incubation period. The incorporation into acceptor glycoprotein was 2-6 times the incorporation into endogenous acceptor. Some properties of the transferase activity are presented in Table 1.

In order to demonstrate the specificity of the enzyme, [^{14}C]-sialylated antifreeze glycoproteins were prepared using solublized enzyme preparations. The product was extensively repurified by selective precipitation of rat liver proteins with 5% trichloroacetic acid, followed by dialysis and chromatography on Sephadex G-50 to remove CMP-[^{14}C]-NANA. As shown in Table 2, essentially all the [^{14}C]-NANA was removed by treatment of 40 µg (400 cpm) quantities of this material with Clostridium perfringens neuraminidase (Worthington) using standard conditions (8) except that the reaction was terminated by addition of 5% phosphotungstic acid in 0.1 N HCl and the products counted as described above. The degree of substitution of disaccharide residues (Fig. 1a) in the preparation (0.002%) is too small to be expected to affect the antifreeze activity.

Figure 1

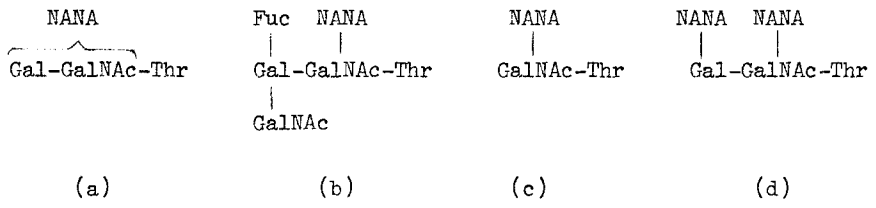

Fig. 1. Structures of the glycoside moieties of (a) sialylated antifreeze glycoproteins, (b) pig submaxillary A mucin, (c) ox and sheep submaxillary mucin, and (d) human red cell MN antigen. The glycoside moieties in (b), (c), and (d) may also be attached to serine.

In order to test how widespread the NANA transferase enzyme is in rats a different type of tissue, a transplantable, chemically induced mammary adenocarcinoma (4),was tested for the presence of the same type of activity. It was possible to solublize NANA transferase activity from excised tumor tissue by a method similar to that employed for the liver activity. A partial characterization of the enzyme activity is presented in Table 1 and compared with the activity solublized from liver.

DISCUSSION

The tendency for sialyltransferases from different organs to differ in their acceptor specificity is well documented (9,10). In this context, the properties of the NANA transferase activities from rat liver and tumor tissue presented in Table 1 are sufficiently similar to suggest that probably it is the same enzyme(s) in both types of rat tissue. Presumably both types of tissue also have NANA acceptors with structures similar to the sugar structure on the antifreeze glycoproteins. These NANA acceptors are probably intermediates in the biosynthesis of sialic acid containing structures such as those found in mucins (11) (Fig. 1b and 1c), in which sialic acid residues are required to impart the characteristic viscosity (9), and blood group substances (11) (Fig. 1d) in which mutual repulsion of the negatively charged sialic

acids are believed to impart strength and rigidity to the red blood cell membrane (9). The presence of similar sugar structures in two divergent tissues of one species and in two divergent species suggests that, in order to be conserved through both tissue differentiation and evolutionary development, they must have a necessary and widespread function. Similarly, the non-freezing adapted evolutionary progenitor of T. borchgrevinki may also have had these sugar structures. This fish may have developed freezing resistance by adapting such a glycoprotein structure to the new role of preventing freezing in serum and tissues by losing the capacity to add on the terminal sialic acid residues - an easier evolutionary development than producing a complete, new synthetic system.

It may also be noted that the data presented in Table 2 indicate that [^{14}C]-sialylated antifreeze glycoproteins are potentially useful as a substrate for a highly sensitive assay for neuraminidase. This assay would have a sensitivity approximately 100 times as great as the method of Warren (12), and comparable to the method of Bernacki and Bosmann (13), but possessing the advantage that it does not employ a substrate analog.

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