A guanosine diphosphate-L-fucose glycoprotein fucosyltransferase from Corynebacterium insidiosum
by Peter Ludwig Sadowski

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology
Montana State University

Abstract:
Since Corynebacterium insidiosum produces a high molecular weight phytotoxic glycoprotein of which 38.7% is fucose, the enzyme catalyzed transfer of fucose to glycoproteins was investigated. Fucosyltransferase activity was demonstrated using a crude cell membrane preparation from C, insidiosum. Guanosine diphosphate-L-fucose was used as the sugar nucleotide glycosyl donor. Two glycoproteins were used as fucosyl acceptors: a phytotoxic glycoprotein from C. insidiosum subjected to mild acid hydrolysis, and a glycosidase-treated αracid glycoprotein obtained from pooled human plasma. The cell membrane preparation demonstrated a pH optimum of 7.5, and the incorporation appeared to be linear for 1+5 minutes. When the bacterial glycoprotein was used, concentrations in excess of 0.2 µg per µ1 of reaction mixture appeared to be saturating. The addition of chelating agents and nonionic detergents inhibited transferase activity. The presence of MnCl2 in the reaction mixture appeared to enhance the incorporation of fucose. The fucosyltransferase could be demonstrated in the soluble fraction.

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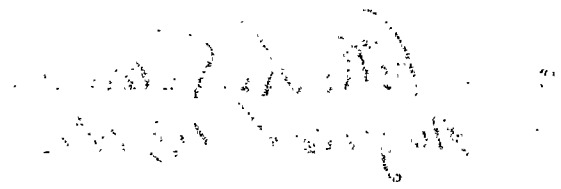

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ABSTRACT

Since Corynebacterium insidiosum produces a high molecular weight phytotoxic glycoprotein of which 38.7% is fucose, the enzyme catalyzed transfer of fucose to glycoproteins was investigated. Fucosyltransferase activity was demonstrated using a crude cell membrane preparation from C. insidiosum. Guanosine diphosphate-L-fucose was used as the sugar nucleotide glycosyl donor. Two glycoproteins were used as fucosyl acceptors: a phytotoxic glycoprotein from C. insidiosum subjected to mild acid hydrolysis, and a glycosidase-treated α_1-acid glycoprotein obtained from pooled human plasma. The cell membrane preparation demonstrated a pH optimum of 7.5, and the incorporation appeared to be linear for 45 minutes. When the bacterial glycoprotein was used, concentrations in excess of 0.2 µg per µl of reaction mixture appeared to be saturating. The addition of chelating agents and nonionic detergents inhibited transferase activity. The presence of $MnCl_2$ in the reaction mixture appeared to enhance the incorporation of fucose. The fucosyltransferase could be demonstrated in the soluble fraction.

INTRODUCTION

In the past decade, membrane associated enzyme systems have been described and implicated in the synthesis of macromolecules in general, and glycoproteins in particular. This work has been reviewed extensively (10,23,28).

The 6-deoxy-L-galactose, L-fucose, is found as a constituent of numerous glycoproteins. It has been found in plasma glycoproteins (7,27), blood group substances (32), glycoproteins of whole brain fractions and nerve endings (18,33), phytohemagglutins from seeds (8), plasma membrane glycoproteins (1), and phytotoxic glycoproteins (25,29).

The transfer of fucose into glycoproteins is catalyzed by a class of enzymes known as fucosyltransferases. Guanosine diphosphate fucose serves as the glycosyl donor in these reactions. The importance of this sugar nucleotide as the glycosyl donor was first demonstrated in vivo by Kornfield et al. (20). They had attempted to detect a nucleotide linked, fucose containing, oligosaccharide intermediate in the synthesis of the heterosaccharide chains of blood group substances. Their attempt to demonstrate the oligosaccharide nucleotide was unsuccessful. They did find large amounts of guanosine diphosphate fucose in the cell fraction, which was shown to contain the newly synthesized blood group substances.

The first workers to demonstrate an enzymatic transfer

of L-fucose from guanosine diphosphate-L-fucose used lactose as the fucosyl acceptor (12). The product of the reaction was the trisaccharide, fucosyllactose, which is the simplest fucose containing oligosaccharide found in milk. The enzyme which catalyzed this reaction was the particulate cell fraction of lactating canine mammary tissue. This particulate preparation would also catalyze the formation of the trisaccharide when incubated with D-glucose, uridine diphosphate-D-galactose, and guanosine diphosphate-L-fucose.

The in vivo incorporation of fucose into the glycoproteins of adult male rats was studied at length by Bekesi and Winzler (3) in 1967. They found that L-fucose-1-^{14}C was a suitable precursor in their examination of the biosynthesis of the glycoprotein as the fucose was not rapidly converted into other sugars or CO_2. By studying the trichloroacetic acid soluble and insoluble radioactive products from animals sacrificed at various times, the following sequence of reactions was postulated:

Fucose $\longrightarrow$ Fucose-1-P $\longrightarrow$ GDP-Fucose $\longrightarrow$ Glycoproteins

More recently, Trujillo and Gan (31) have shown that the sequence of reactions proposed by Bekesi and Winzler can also be predicted from in vitro studies using bovine thyroid gland slices. The in vitro incorporation of fucose-1-^{14}C was

similarly followed by chromatographic analysis of the trichloroacetic acid soluble and insoluble radioactive products found at various times during incubation.

Grollman and Marcus (13) demonstrated the in vitro incorporation of fucose into soluble blood group H substance. The glycosyl donor was guanosine diphosphate-L-fucose, and the enzyme preparation was obtained from hog gastric mucosa. An immunological technique was available for the identification of the radioactive endproduct as the receptor plus incorporated fucose was identical to the blood group H substance, the H specificity being determined by the terminal α-L-fucosyl-(1$\rightarrow$2)-β-D-galactosyl linkage.

The antigenic determinants of the A, B, O(H) system all have fucose α-(1$\rightarrow$2) linked to a D-galactosyl residue (26). However, the distinguishing feature between the Lewis blood group type a (Le^a) and the Lewis blood group type b (Le^b) is that the Le^b glycoprotein has a fucose (1$\rightarrow$2) linkage whereas the Le^a glycoprotein does not (14).

Using this information and the fact that "secretors" secrete A, B, O(H) soluble blood group substances and "nonsecretors" secrete Le^a glycoprotein, Shen et al. (26) demonstrated that blood group substance specificity can be accounted for by a specific fucosyltransferase. From the information about the

fucosyl linkages in antigenic determinants of the various blood groups, it could be predicted that one should find a specific α-(1$\longrightarrow$2)-fucosyltransferase in "secretors" and an absence of this transferase in "nonsecretors". This was established to be the case.

Further work on α-L-fucosyltransferases and their relationships to blood group characters was done by Chester and Watkins (6) and by Grollman et al. (14) in 1969. In the former paper, 2-, 3-, and 4-α-L-fucosyltransferases were demonstrated in persons who are "secretors" of soluble blood group substances; "nonsecretors" were found to have 3- and 4-α-L-fucosyltransferases. The latter paper distinguishes enzymatically between women who are $Le^a(+)$ or $Le^b(+)$ and women who are $Le^a(-)$ or $Le^b(-)$. The structural determinants of Le^a and Le^b contain fucose linked α-(1$\longrightarrow$4) to N-acetylglucosamine. An α-(1$\longrightarrow$4)-fucosyltransferase was demonstrated in the milk of women who had Lewis type antigens. More recently, Jarkovsky (17) also did some work on fucosyltransferases in human milk which were specified by the Lewis blood type.

During this time, not all work on fucosyltransferases was done using the blood group substances. In 1968, Bosmann et al. (5) found two fucosyltransferases in HeLa cells. The

objections to some of the previous work on transferases using particulate enzyme preparations was avoided in this work by solubilizing the transferases with the nonionic detergent, Triton X-100. The evidence for two fucosyltransferases is based on specificity. Two primers showed successful incorporation of fucose: fetuin, from which sialic acid and galactose were removed exposing a terminal N-acetylglucosamine, and porcine submaxillary glycoprotein from which sialic acid and fucose were removed exposing a terminal galactose. Glycoprotein fucosyltransferase activity was sought in HeLa cells since 94% of the fucose in the cell is not extractable by organic solvents and therefore presumably bound in glycoproteins.

In the early 1970's, fucosyltransferases responsible for the incorporation of fucose into glycoproteins have been found in many systems. Grimes and Burge (11) have identified a fucosyltransferase in chick embryo fibroblast cultures responsible for the incorporation of fucose into the membrane glycoprotein of Sindbis virus. This activity can be demonstrated in infected and uninfected cells implying some sort of host modification. A fucosyltransferase has been isolated from rat small intestine mucosa responsible for α-(1$\rightarrow$2) linkages in blood groups (4). Jabbal and Schachter (16) have found two fucosyltransferases in pork liver. They are differentiated not only by

primer specificity, but also by kinetic data. Parkhouse and Melchers (24) and Melchers (22) have found a fucosyltransferase responsible for the synthesis of the carbohydrate portion of immunoglobulin M (IgM) obtained from mouse plasma-cell tumor MOPC 21. The incorporation of fucose was necessary for the secretion of IgM. Zatz and Barondes (33,34) have identified fucosyltransferases in rat brain which will incorporate fucose into porcine plasma glycoproteins after they have been subjected to mild acid hydrolysis as well as into endogenous receptors.

No bacterial fucosyltransferases have been reported even though Ginsburg has identified an enzyme from Aerobacter aerogenes responsible for the formation of guanosine diphosphate fucose (9).

Since Corynebacterium insidiosum produces a high molecular weight phytotoxic glycoprotein of which 38.7% is fucose (25), it would seem reasonable that this bacterium possesses guanosine diphosphate-L-fucose fucosyltransferase activity. Therefore, the purpose of this report is twofold: 1) to demonstrate fucosyltransferase activity (Fig. 1), and 2) to establish some of the parameters of this activity.

Figure 1. Scheme of guanosine diphosphate-L-fucose fucosyltransferase activity from C. insidiosum using partially hydrolyzed phytotoxic glycoprotein as the primer.

GDP-L-FUCOSE+PRIMER+
CEII MEMBRANE ENZYME →

PRIMER-L-FUCOSE+GDP+
CEII MEMBRANE ENZYME

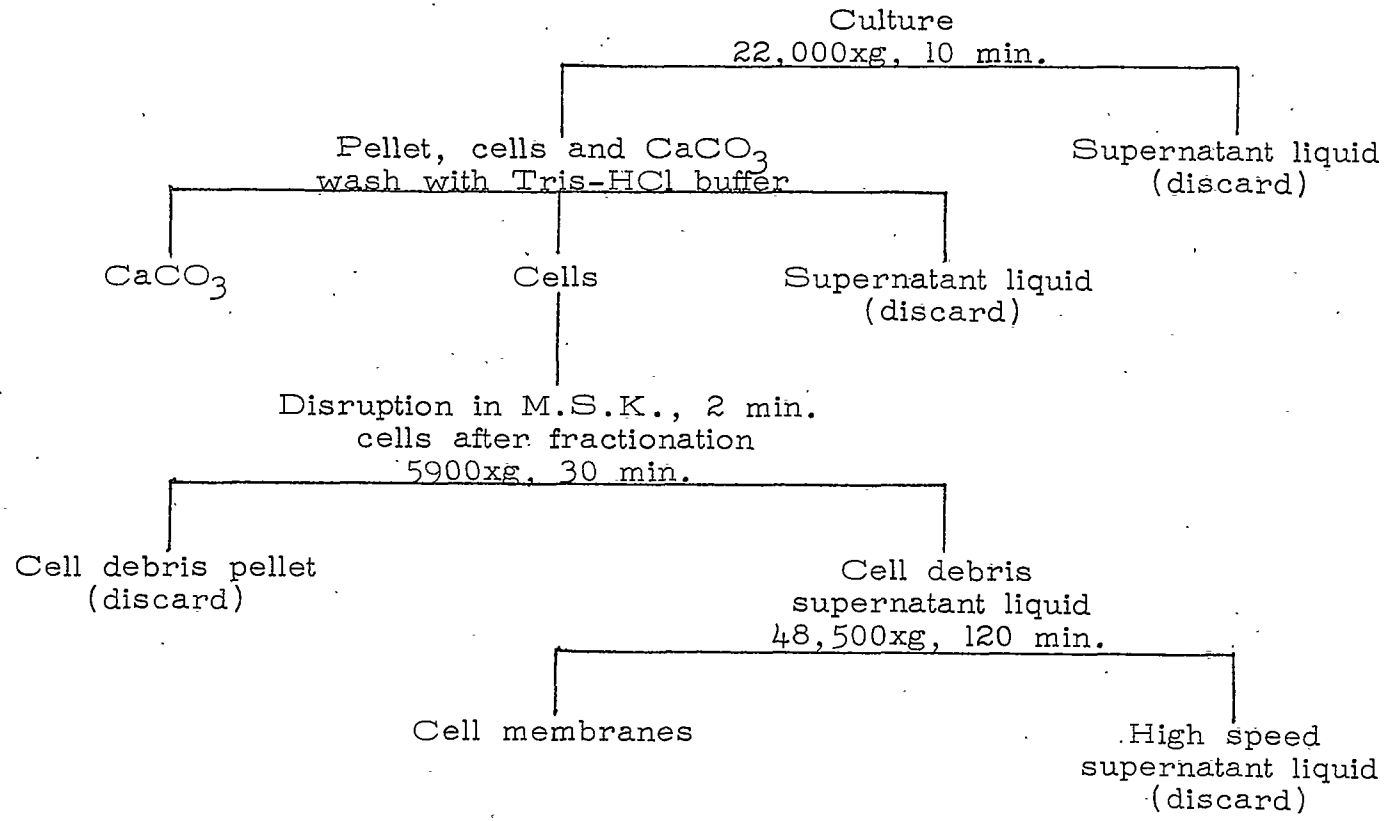
EXPERIMENTAL PROCEDURE

Culturing: The culture of Corynebacterium insidiosum (McCull.) Jensen (courtesy of F. I. Frosheiser, University of Minnesota) was maintained and cultured as described by Ries and Strobel (25) except that sucrose was substituted for glucose as the carbon source.

Preparation of cell free extract: The cells were harvested at specified times. The cell membranes and other subcellular fractions were obtained as described in Table 1. The cells were collected by subjecting the suspension to centrifugation at 22,000xg and 4°C. Subsequent steps were conducted at 4°C unless otherwise specified. The cells were washed 3-5 times with a buffer which consisted of Tris-HCl, 0.05M, pH 7.5 and 0.4M sucrose. The cells were disrupted in an Apparatebau Melsungen (referred to as M.S.K.) using glass beads (Glasperlen, 0.11-0.12 mm) previously washed in buffer. The cells were disrupted in 20 second bursts to a total of 2 minutes to avoid overheating. The cell membrane pellet, obtained by subjecting the cell debris supernatant liquid to centrifugation at 48,500xg for 120 minutes, was resuspended in a solution consisting of 0.05M Tris-HCl, and 0.4M sucrose, pH 7.5, using a 15 ml Pyrex glass homogenizer.

Protein determination: Protein was determined by the method of

Table 1. The fractionation scheme for C. insidiosum



Lowry et al. (21). Bovine serum albumin was used as a standard.

Preparation of primer: The phytotoxic glycoprotein from C. insidiosum was obtained as described by Ries and Strobel (25). The toxin was stored at -20°C until used. The primer was routinely prepared by dissolving 100 mg lots of glycoprotein in 2N HCl, 3-5 ml, in a sealed test tube and heating the solution in an oven at 100°C for 30 minutes. The product was dialyzed against frequent changes of distilled deionized water at 4°C for a minimun of 72 hours. The glycoprotein which came out of solution was removed by centrifugation and the resulting supernatant dried using hot air. When primer was needed, the dried supernatant was resuspended in distilled deionized water. The glycosidase-treated α_1-acid glycoprotein was a gift from Dr. H. Schachter, Department of Biochemistry, University of Toronto.

Substrate: Guanosine diphosphate-L-fucose-^{14}C(U), (GDP-fucose-^{14}C), ammonium salt, 195 mCi/mmol, was obtained from Amersham/Searle and stored at -20°C until used. The solution used for enzyme assay was prepared by dissolving the GDP-fucose-^{14}C in distilled water (55,500 cpm/µl; 128 p moles).

Fucosyltransferase assay: The enzyme preparation, primer,

and substrate were combined on a parafilm strip and then incubated in a small diameter glass tube at 20°C. The concentration of the various reactants was as specified. The reactions were terminated by spotting the reaction mixtures on Whatman no. 1 paper. The unreacted substrate was removed by high voltage paper electrophoresis at 24 v/cm in 0.05M sodium tetraborate, pH 9.1. Under these conditions, the substrate (and breakdown products of the substrate) will migrate but polymeric products will remain at the origin. The amount of fucose incorporated into glycoprotein was determined by cutting out the origin and measuring the radioactivity in a Packard Liquid Scintillation Spectrometer (Model 3320). The origin strip was immersed in a liquid scintillation cocktail consisting of 1.5 ml of absolute methanol and 13.5 ml of scintillation solution containing 5.0 g of 2,5-diphenyloxazole and 100 mg of p-bis-2(5-phenyloxazolyl)-benzene per liter of toluene. The channels ratio method was used to correct for quenching.

One experiment was conducted using a Sephadex G-25 column to separate the unreacted substrate from the reaction mixture. The reaction was incubated in a small test tube at 20°C and 35 revolutions per minute. The reaction was terminated by placing the test tube in a boiling water bath and drying the reaction mixture with a stream of nitrogen gas. The

product mixture was resuspended in 400 µl of distilled water and fractionated by chromatography on a Sephadex G-25 column, 31 cm x 1.9 cm. The 0.5 ml fractions were collected, and then placed in counting vials containing 15 ml of Aquasol universal liquid scintillation cocktail (New England Nuclear).

Verification of incorporation of fucose: The reaction products were eluted from the origin with 10 ml of water per origin after electrophoresis of 13 standard reaction mixtures. The eluant was collected and dried using hot air, and then further dried under vacuum over P_2O_5 at room temperature. The radiolabeled glycoprotein was dissolved in distilled water to give a final concentration of 3640 dpm in a 100 µl. A 30 µl aliquot of glycoprotein was dried in a test tube and resuspended in 2.0 ml of 1N TFA (trifluoroacetic acid) and subjected to hydrolysis in a sealed test tube for 2 hours at 120°C.

Sugars were identified by descending paper chromatography in ethyl acetate-acetate-formic acid-H_2O (18:3:1:4) v/v/v/v. The sugar standards were located using the methods of Trevelyan (30). The radiolabeled primer and monosaccharides were located using a Packard Radiochromatogram Scanner (Model 385).

Materials: Triton X-100 and tris(hydroxymethyl)aminomethane

(Trizma) were obtained from Sigma Chemical Company. The sodium cacodylate and the EDTA were obtained from K&K Laboratories, Inc. All other chemicals used were reagent grade.

RESULTS

Products of the reaction: Electrophoresis of any reaction mixture containing primer and active enzyme yielded two radioactive peaks. One was at the origin and the other was a broad peak extending 10 cm to 16 cm off the origin relative to the movement of the picric acid marker at 20 cm. The components which remained at the origin were subjected to descending paper chromatography following elution. The results are shown in Figure 2. Chromatography of the unhydrolyzed product showed all the radioactivity at the origin. The hydrolyzed reaction product showed a trace of radioactivity at the origin and a majority of the radioactivity approximately four inches from the origin. Only trace amounts of the radioactivity incorporated appeared to be soluble in the liquid scintillation cocktail.

Location of transferase activity in the subcellular fractions: Subcellular fractions were assayed for fucosyltransferase activity. The results from a typical cell preparation procedure are shown in Table 2. The high speed supernatant liquid demonstrated a higher specific activity than the cell membrane precipitate, however, inasmuch as the activity associated with the membrane fraction could be easily concentrated by pelleting at 48,500xg, it was used as a subject of this study. Dr. William Hess kindly supplied the electron micrograph (Fig. 3) of the cell

Figure 2. Tracing of radiochromatograms of sugar residues found in unhydrolyzed (X) and hydrolyzed (Y) phytotoxic glycoprotein primers. Each complete reaction mixture contained: phytotoxic glycoprotein subjected to mild acid hydrolysis, 3.7 µg; GDP-fucose-^{14}C, 26.2 p moles; Tris, pH 7.5, 1.25 micromoles; sucrose, 10 micromoles; and 20 µg of enzyme protein in a total volume of .035 ml. The cells were cultured for 50 hours prior to being harvested.