Multiglycosyltransferase System of Canine Respiratory Tissue

URIDINE DIPHOSPHATE GALACTOSE: MUCIN GALACTOSYLTRANSFERASE*

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SUMMARY

A galactosyltransferase has been found in a particulate preparation prepared from normal canine respiratory tissue which catalyzes the transfer of galactose from its uridine diphosphate derivative to N-acetylgalactosamine residues. The transfer to sialidase-treated ovine submaxillary mucin was dependent upon Mn** and was stimulated several-fold by Triton X-100. The optimal pH was between 6 and 7. The K_m was 7 x 10^{-4} M for UDP-galactose and 1 x 10^{-2} M for sialidase-treated ovine submaxillary mucin. Removal of the N-acetylgalactosamine residues on this acceptor reduced the incorporation of galactose. The enzyme was present throughout the airway passage with some evidence of higher activity in the lower portion of the trachea and the extrapulmonary primary bronchi.

Fetuin from which sialic acid and galactose were removed was also an acceptor (K_m = 0.9 x 10^{-3} M). When the terminal N-acetylgalactosamine residues were removed from this acceptor, the incorporation of galactose was reduced. No competition was demonstrated when this acceptor was mixed with sialidase-treated ovine submaxillary mucin; this suggests that there are at least two separate galactosyltransferases present in canine respiratory tissue or two catalytic sites on the same molecule.

EXPERIMENTAL PROCEDURE

Glycoproteins are the major macromolecular components of the secretion elaborated by the mucus-producing structures of the tracheobronchial tree. Those isolated from both human and canine respiratory secretions have the characteristic structural patterns of other mucin-type glycoproteins. The small polysaccharide units glycosidically linked to the protein core constitute about 40% of the molecular weight and include sialic acid, fucose, galactose, and N-acetylgalactosamine. The studies of Gernet-Rieux et al. (2) indicate that in human bronchial mucin, a basic unit made up of galactose, N-acetylgalactosamine, and N-acetylgalactosamine residues is attached to the

UDP-Gal + GalNAc → protein

Gal → GalNAc → protein (+UDP)

Materials—UDP-[U-^{14}C]galactose and [U-^{14}C]galactose were purchased from New England Nuclear, and the corresponding nonradioactive substances obtained from Calbiochem were used to adjust the specific activity. [U-^{14}C]Galactose-1-phosphate was purchased from International Chemical and Nuclear Corporation. UDP-N-acetyl-[U-^{14}C]glucosamine and nonradioactive UDP-N-acetylglucosamine were obtained from New England Nuclear and Boehringer Mannheim, respectively. Fresh-frozen submaxillary glands were obtained from Pentex, Inc., Kanakee, Illinois, and fresh-frozen canine trachea were

* A preliminary report of this work was reported at the meeting of the American Society of Biological Chemists in April 1970 (1).

1 R. Hayes and G. Biserte, Abstracts of papers presented at the Colloque International de Pathologie Thoracique, Lille, France, September 27 to 29, 1968, p. 43.

2 K. Holden and L. Grigg, personal communication.
purchased from Rockland. Adult male standard laboratory beagles that were normal by clinical and laboratory studies were supplied by White Eagle Farm. Ovine, bovine, and porcine submaxillary mucins were isolated essentially by the procedure of Hashimoto, Hashimoto, and Pigman (6) as modified by De-Sualegui and Pigman (7). Pig submaxillary glands were pooled according to their ability to inhibit human A-anti A hemagglutination (8). Fetuin was prepared as previously described by Spiri (9). Sialic acid residues were removed (over 95%) by deamination (8). Fctuin was prepared as previously described by Carlson, McGuire, and Jourdian (10). N-Acetyl-
galactosamine and galactose residues were removed by digestion with N-acetylgalactosaminidase and galactosidase, respectively, obtained from a strain of Clostridium perfringens (11). The exact experimental details for the preparation of these glycosidases were kindly supplied by Mr. S. Chipowsky and Dr. E. McGuire. The enzymes were purified between 500- and 1000-fold and were free of any proteolytic activity. Approximately 50 mg of the glycoprotein, dissolved in 12 ml of 0.1 M phosphate buffer, pH 6.0, were treated with approximately 1 unit of the appropriate glycosidase. Over 95% of the protein-bound N-acetylglactosamine residues were removed, whereas only 70% of the galactose residues could be removed from Fet(-NAN). Unless stated differently, the concentrations of all glycoproteins are expressed as the number of positions available for sugar attachment on each of the modified glycoproteins. This number is calculated from the number of sugar residues released by the respective glycosidase. Methyl 2-acetamido-2-deoxy-a-D-galactopyranoside was generously supplied by Dr. Lee Griggs. All other chemicals used were reagent grade and were used without further purification.

Gas Chromatography—Carbohydrate analyses by gas chromatography were performed as described by Griggs (12).

Paper Chromatography—Descending paper chromatography was performed on Whatman No. 1 paper in Solvent A, ethyl acetate-glacial acetic acid-water, 140:50:30 (v/v); or Solvent B, 1-butanol-pyridine-water, 6:4:3 (v/v). Radioautography using Kodak Royal Blue X-ray film was used to visualize chromatograms for radioactivity.

Preparation of Particulate Enzyme—All procedures in the preparation of the particles were performed at 0-4°C. The inner mucosal lining of fresh-frozen canine tracheas were removed by scraping with a scalpel, and were homogenized in a Virtis homogenizer in 8 volumes of 0.25 M sucrose containing 0.001 M EDTA and 0.01 M 2-mercaptoethanol at pH 7.4. The homogenate was centrifuged at 12,000 x g for 15 min, yielding a supernatant which was subsequently centrifuged at 105,000 x g for 1 hour. The resulting particles were washed twice with the sucrose solution, using 5 volumes per g of original scrapings, and centrifuged at 105,000 x g for 1 hour after each wash. The washed particles were resuspended in the sucrose solution (0.2 ml per g of original scrapings) and stored at -20°C. The high speed supernatants had no enzymatic activity. These preparations were stable to freezing and thawing, and over a period of several months there was no significant loss of activity.

Assay of Enzymatic Activity—The galactosyltransferase assay was performed at 37° in a final volume of 0.05 ml, contain-

4The abbreviations used are: Fet(NAN, Gal), sialidase-galactosidase-treated fetuin; OSM(-NAN), sialidase-treated ovine submaxillary mucin; NAN, N-acetylneuraminic acid.

65 mg of sialidase-treated ovine submaxillary mucin, 0.05 μmoles of UDP-galactose (100 dpn per μmole), 2.5 μmoles of 2-(N-morpholino)ethane sulfonic acid, pH 6.5, 0.01 ml of 8% Triton X-100, 1.5 μmoles of MnCl₂, and enzyme. After the mixture was incubated for 60 min, 0.01 ml of 0.3 M EDTA was added to terminate the reaction. An aliquot of the incubation mixture was subjected to paper electrophoresis on Whatman No. 3MM paper saturated with tetraborate, pH 9.0, for 30 min (13). The labeled substrate and its degradation products migrated rapidly, whereas the product remained at the origin. Areas of the paper at the origin were counted by liquid scintillation methods (Packard Tri-Carb), using a toluene system prepared by adding 74 ml of the concentrated liquid scintillator, Spectrafluor butyl-PBD (Amersham-Searle), to 1 liter of toluene. The efficiency for counting ¹⁴C under these conditions was approximately 75%.

Protein Determination—Protein was estimated by the method of Lowry et al. (14) using crystalline bovine serum albumin as a standard.

Estimation of Kₘ and v max—Kₘ and v max were determined by a computer-generated linear regression analysis by the method of Wilkinson (15).

RESULTS

Requirements of Enzymatic Activity—The requirements for the incorporation of galactose into OSM(-NAN) are shown in Table I. Mn²⁺ was essential for activity, while other divalent cations tested showed either little or no activity. The reaction was stimulated approximately 4-fold by Triton X-100. The low activity in the absence of OSM(-NAN) suggested that only a small amount of endogenous acceptors was present in the particulate enzymatic preparation.

Kinetic Studies—The transfer of galactose was linear with time for at least 4 hours and was proportional to protein concentration from 0.65 to 3.4 mg per ml of assay mixture. The enzyme displayed optimum activity in 2-(N-morpholino)ethane sulfonic acid buffer between pH 6 and 7 (Fig. 1). Transfer of galactose to OSM(-NAN) as a function of nucleotide concentration is illustrated in Fig. 2. An approximate Kₘ value of 7 x 10⁻⁸ M was determined for UDP-galactose. The effect of increasing the concentration of OSM(-NAN) on the enzymatic activity is summarized in Fig. 3. An approximate Kₘ value for OSM(-NAN) was calculated to be 1 x 10⁻⁴. Fig. 4 shows the dependence of

TABLE I

Requirements of canine tracheal UDP-galactose: mucin galactosyltransferase

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Galactose transferred (nmoles/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>166</td>
</tr>
<tr>
<td>Minus OSM(-NAN)</td>
<td>3</td>
</tr>
<tr>
<td>Minus enzyme</td>
<td>2</td>
</tr>
<tr>
<td>Minus Mn²⁺</td>
<td>2</td>
</tr>
<tr>
<td>Minus Mn²⁺ plus Mg²⁺</td>
<td>7</td>
</tr>
<tr>
<td>Minus Mn²⁺ plus Ca²⁺</td>
<td>7</td>
</tr>
<tr>
<td>Minus Mn²⁺ plus Zn²⁺</td>
<td>2</td>
</tr>
<tr>
<td>Minus Triton X-100</td>
<td>38</td>
</tr>
<tr>
<td>Minus enzyme plus boiled enzyme</td>
<td>2</td>
</tr>
</tbody>
</table>
to the 14C-product was pooled and lyophilized. The residue will be referred to as [14C]mucin product. Electrophoresis of this [14C]mucin product gave only one radioactive spot at the origin in 1% borate, pH 9.0.

A portion of the [14C]mucin product was hydrolyzed in 2 N HCl at 100° for 90 min, the acid was removed, and the residue was reconstituted in water. Aliquots were subjected to electrophoresis in 1% borate (pH 9.0) and chromatography in Solvents A and B, and in each case, only one radioactive spot was found which corresponded to galactose.

To characterize further the nature of the linkage between the incorporated galactose and OSM(NAN), 2 μmoles of the [14C]mucin product was treated with alkaline borohydride (8). After the mixture was neutralized with acetic acid, it was passed through a column of Dowex 50 X12, 200 to 400 mesh, H+ form, to remove cations. The eluate was evaporated to dryness, and borate was removed by repeated evaporation in the presence of methanol. The residue containing the reduced oligosaccharide was subjected to paper chromatography in Solvents A and B and electrophoresis in 1% borate, pH 9.0. Only a single radioactive band which migrated differently from the [14C]mucin product was observed. After hydrolysis of the reduced oligosaccharide in 2 N HCl at 100° for 90 min, the acid was removed and the hydrolysate was subjected to electrophoresis in 1% borate, pH 9.0, and paper chromatography in Solvents A and B. Only one radioactive spot was detected which corresponded to galactose.

Carbohydrate analysis of the [14C]mucin product by gas chromatography showed the presence of only two sugar components. The retention times of these components were identical with those of galactitol hexaacetate and N-acetylglactosaminitol pentaacetate.

Treatment of both the mucin and the reduced disaccharide with galactosidase from C. perfringens, galactosidase from Escherichia coli (Worthington Biochemical Corporation), or galactosidase from Aspergillus niger (16) failed to release galactose as evidenced by the electrophoretic pattern following incubation. These enzymes have been shown to release galactose in β(1→3), β(1→4), and α(1→6) linkages, respectively.

Specificity of Galactosyltransferase—When [U-14C]galactose, [U-14C]galactose 1-phosphate, or UDP-N-acetyl[1-14C]glucosamine were incubated with OSM(NAN) instead of UDP-[U-14C]galactose in the standard assay, there was no incorporation of the labeled sugar.

The particulate enzymatic preparation prepared from canine tracheal tissue was capable of transferring galactose to several acceptors, as shown in Table II. A comparison of the Vmax values and Km values suggests that glycoproteins containing terminal N-acetylgalactosamine or N-acetylgalactosamine were the best acceptors. When N-acetylgalactosamine and N-acetylgalactosamine were removed from OSM(NAN) and Fet(NAN, Gal), respectively, the amount of galactose incorporated was markedly reduced. Inactive acceptors were α- and β-methyl-D-glucoside, N-acetyl-d-mannosamine, α-methyl-D-mannosamine, and porcine submaxillary mucins (A+ and A-).

Effect of Mixed Substrates on Enzymatic Activity—The ability of the enzyme preparation to transfer galactose to N-acetylgalactosamine, N-acetylgalactosamine, and glucose in the presence of α-lactalbumin suggests the presence of more than one catalytic center. Mixed substrate experiments were performed and the results are summarized in Table III. The transfer of
galactosamine did not show a summation of activity, indicating that the transfer to these two acceptors is due to the same enzyme. The competition seen with a mixture of Fet(-NAN, Gal), N-acetylgalactosamine, or glucose suggests that a single enzyme acted on these acceptors. α-Lactalbumin was necessary for the transfer of galactose to glucose but markedly inhibited transfer to free N-acetylgalactosamine and also, to a lesser extent, to Fet(-NAN, Gal) and OSM(-NAN).

Distribution of Enzymatic Activity in Respiratory Tract—The respiratory tract was removed from anesthetized normal dogs and sections representing the following areas were removed: (a) cranial cervical trachea, (b) caudal cervical trachea, (c) extra-pulmonary primary bronchi, (d) intrapulmonary bronchi, (e) peripheral lung.

The enzymatic particulate preparations were immediately prepared from each section and assayed for UDP-galactose:mucin galactosyltransferase activity as described in the text. In this study, not just the mucosal lining but a transverse section of the trachea was homogenized. The results are summarized in Table IV. The enzyme was present throughout the airway passage with some evidence of higher activity in the lower portion of the trachea and the extrapulmonary primary bronchi.

DISCUSSION

It is believed that the biosynthesis of the oligosaccharide side chains of glycoproteins involves a series of glycosyltransferases which attach 1 monosaccharide residue at a time to the growing unit (5). A galactosyltransferase has been demonstrated in normal canine tracheal tissue which is consistent with such a hypothesis. The enzyme catalyzes the transfer of galactose residues to terminal N-acetylgalactosamine residues of mucin type molecules; removal of the N-acetylgalactosamine residues results in a marked decrease of enzymatic incorporation of galactose. Free N-acetylgalactosamine, as well as the α-methyl glycoside of this hexosamine, also served as acceptors.

The mucin galactosyltransferase from canine trachea appears to be similar in many respects to the galactosyltransferase from porcine submaxillary gland (17, 18). Both have the same acceptor specificity. The transfer is dependent upon Mn++ and is stimulated several-fold by Triton X-100. The kinetic constants of tracheal galactosyltransferase are similar to those reported for the submaxillary enzyme; differences are probably due to the particular nature of both preparations. Unlike the galactosyltransferase from porcine submaxillary gland, the in-

### Table II

**Substrate specificity of canine tracheal galactosyltransferase**

The standard assay as described in the text was used, using 25 μg of enzyme protein. When glucose was the acceptor, 0.25 nmole of UTP and 50 μg of α-lactalbumin were added to the incubation mixture.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Galactose transferred</th>
<th>Vmax</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine submaxillary mucin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>66</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>NAN-free</td>
<td>290</td>
<td>339</td>
<td>0.6</td>
</tr>
<tr>
<td>Ovine submaxillary mucin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>23</td>
<td>54</td>
<td>8.7</td>
</tr>
<tr>
<td>NAN-free</td>
<td>110</td>
<td>155</td>
<td>1.3</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>90</td>
<td>100</td>
<td>3.4</td>
</tr>
<tr>
<td>N-acetylgalactosamine</td>
<td>15</td>
<td>142</td>
<td>210</td>
</tr>
<tr>
<td>Methyl-2-acetamido-2-deoxy-α-n-galactopyranoside</td>
<td>147</td>
<td>170</td>
<td>3.4</td>
</tr>
<tr>
<td>Fetin</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAN-free</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>80</td>
<td>141</td>
<td>0.9</td>
</tr>
<tr>
<td>glucosamine</td>
<td>62</td>
<td>73</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* Concentration is expressed as milligrams, dry wt, of the acceptor and not as acceptor site concentration.

### Table III

**Mixed substrate experiments**

The standard assay as described in the text was used, using 127 μg of enzyme protein and the following amounts of acceptor molecules (micromoles): OSM(-NAN), 0.25; Fet(-NAN, Gal), 0.05; N-acetylgalactosamine, 0.45; N-acetylgalactosamine, 22.6; glucose, 0.55. α-Lactalbumin concentration was 0.1%. The mucin galactosyltransferase from canine trachea appears to be similar in many respects to the galactosyltransferase from porcine submaxillary gland (17, 18). Both have the same acceptor specificity. The transfer is dependent upon Mn++ and is stimulated several-fold by Triton X-100. The kinetic constants of tracheal galactosyltransferase are similar to those reported for the submaxillary enzyme; differences are probably due to the particular nature of both preparations. Unlike the galactosyltransferase from porcine submaxillary gland, the in-

### Table IV

**Distribution of UDP-galactose:mucin galactosyltransferase activity in canine respiratory tract**

The assay conditions were the same as described in the text. Of the enzyme preparations, 25 μl of each were used in the assays.

<table>
<thead>
<tr>
<th>Section</th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Dog 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial cervical trachea</td>
<td>4.2</td>
<td>4.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Caudal cervical trachea</td>
<td>3.6</td>
<td>3.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Extrapulmonary primary bronchi</td>
<td>4.6</td>
<td>4.9</td>
<td>5.4</td>
</tr>
<tr>
<td>Intrapulmonary bronchi</td>
<td>5.0</td>
<td>6.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Peripheral lung</td>
<td>7.4</td>
<td>5.6</td>
<td>8.8</td>
</tr>
</tbody>
</table>

* Concentration is expressed as milligrams, dry wt, of the acceptor and not as acceptor site concentration.
corporated galactose was not susceptible to hydrolysis by a galactosidase which hydrolyzes β(1→3) linkages; nor was it susceptible to galactosidases which hydrolyze β(1→4) or α(1→6) linkages. This suggests that galactose is being incorporated by the tracheal enzyme onto terminal N-acetylgalactosamine residues of OSM(-NAN) by a linkage other than these three possibilities. Alternatively, the incorporated galactose may not be susceptible to hydrolysis by the available galactosidases because of stearic hindrance caused by the protein core of ovine submaxillary mucin.

In addition to the mucin galactosyltransferase, mixed substrate experiments (Table III) suggest the presence of either another galactosyltransferase or an additional enzyme site. This enzyme is similar to the galactosyltransferase which is found in colostrum and tissue fractions and which utilizes terminal N-acetylglucosamine positions on glycoproteins (19, 20). Free N-acetylgalactosamine is also an acceptor.

These results suggest that the following sequences are present in canine tracheal mucins: galactosyl-N-acetylgalactosamine and galactosyl-N-acetylglucosamine. Carbohydrate analyses have shown the presence of these sugars in canine tracheal mucins, but no structural studies have been done on the native molecules to confirm these speculated sequences. In addition, two other glycosyltransferases have been demonstrated in normal canine respiratory tissue (21). One is an N-acetylgalactosaminyltransferase which transfers N-acetylgalactosamine residues to a polypeptide, and the other is a sialyltransferase which transfers sialic acid to N-acetylgalactosamine residues.

Based on the composition of the oligosaccharide side chains, three fundamental types of glycoproteins have been characterized from tracheobronchial secretions (2). It appears that certain chronic obstructive lung diseases associated with hypersecretion are characterized, not by abnormal constituents, but by abnormal proportions of these three basic constituents (4). This would indicate an alternation of the relative activities of the glycosyltransferases of the multienzyme system present in respiratory tissue. An irritant-induced state which closely resembles the histopathological characteristics of human chronic bronchitis can be produced in animals by the inhalation of noxious materials (22); present studies are concerned with determining the activities of the glycosyltransferases in this experimentally induced disease state.

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REFERENCES