An Effect of Puromycin on Galactosyltransferase of
Golgi-rich Fractions from Rat Liver

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SUMMARY

Some of the properties of galactosyltransferase of Golgi membrane-rich fractions from rat liver were investigated. The kinetic properties of the enzyme were determined from the initial rates of reaction under various conditions of incubation. Puromycin, a known inhibitor of protein synthesis, was shown to inhibit the galactosyltransferase activity in vitro. The inhibition depended on the concentration of puromycin and on the duration of exposure of Golgi membranes to the drug. A number of compounds, structurally related to puromycin, were unable to produce the inhibition. A combination of the aminonucleoside and amino acid portions of the puromycin molecule was equally ineffective. Binding of puromycin to the membranes was demonstrated with [3H]puromycin. It was concluded that this binding to the membrane disrupted the enzymatic activity.

Most plasma glycoproteins are synthesized by the liver, except immunoglobulins which are synthesized in plasma cells. Glycoproteins are conjugated proteins in which the prosthetic groups are oligosaccharide chains covalently linked to the functional group of the side chain of an amino acid in the protein. In liver, the protein portion of glycoproteins intended for secretion is synthesized on polysomes bound to the rough endoplasmic reticulum and is subsequently transported through the smooth endoplasmic reticulum and the Golgi apparatus (1-3). Addition of carbohydrates during this passage is catalyzed by glycosyltransferase enzymes located in these smooth membranes (4-8). These enzymes catalyze the transfer of a sugar from its nucleotide-sugar donor to an acceptor which is the appropriate side chain of an amino acid in the protein or a sugar in the terminal position of an oligosaccharide side chain.

Galactosyltransferase catalyzes the transfer of galactose from UDP-galactose to an N-acetylglucosamine residue on the oligosaccharide side chain of a partially completed glycoprotein molecule in vitro. The enzyme has been demonstrated in vitro by measuring the transfer of [3H]galactose from exogenous UDP-[3H]galactose to endogenous glycoprotein acceptors containing terminal N-acetylgalactosamine residues (7). The reaction has been shown to be catalyzed by isolated Golgi membranes (6, 7, 9). Golgi membrane-rich fractions also catalyze the transfer of [3H]galactose to exogenous acceptors, desialylatedgalactosyl-
glycoproteins or to N-acetylglucosamine forming N-acetylgalactosamine (4-6).

The inhibition of protein synthesis by puromycin has been shown both in vivo and in vitro (9-14). The mechanism of action of puromycin depends on the structural similarity of puromycin to the terminal aminooacyladenosine residue of aminoacyl-tRNA. It interrupts protein synthesis by preventing peptide chain elongation at the ribosomal level (11, 14-16, 17). In addition to its effect on protein synthesis, however, many other changes in cellular metabolism have been described subsequent to puromycin treatment. Among these effects are the ultrastructural changes in intracellular membranes of rat and mouse hepatocytes, particularly in the endoplasmic reticulum and the Golgi apparatus (18-20), where distorted Golgi sacules and tubules have been described. Ultrastructural changes in the Golgi apparatus have been shown to persist for up to 24 hours, long after protein synthesis and the morphology of the endoplasmic reticulum have returned to normal (18). In vivo studies have also indicated inhibition of incorporation of carbohydrates into glycoproteins in puromycin-treated animals (21-24).

In order to separate the effects of puromycin on protein and glycoprotein synthesis, the isolated Golgi membrane fraction was used to study the actions of puromycin. Previously, the inhibitory effects of puromycin on glycoprotein biosynthesis have been related to a lack of precursors (21, 24, 25). In these studies, a direct effect of puromycin on the galactosyltransferase activity of the Golgi apparatus was demonstrated and investigated.

A necessary requirement for these studies was the establishment of kinetic parameters such as $K_m$ and $V_{max}$ values for galactosyltransferase from rat Golgi. Similar studies have been reported for the pork liver enzyme (5).

MATERIALS AND METHODS

Isolation of Golgi—Golgi-rich fractions were isolated from the livers of male Wistar rats according to the method of Sturgess et al. (26). The purity of each preparation was monitored by electron microscopy and assays for galactosyltransferase. Galactosyltransferase activity was determined according to the method of Schachter et al. (5).

Galactosyltransferase Activity—For the measurement of galactosyltransferase activity toward N-acetylglucosamine (GalNAc), the incubation medium contained 5 $\mu$moles of GalNAc, 0.05 $\mu$ mole of UDP-galactose (UDP-Gal) (Calbiochem) containing 10$^3$ dpm of UDP-[3H]Gal (New England Nuclear), 3 $\mu$moles of MnCl$_2$, 1. J. M. Sturgess and M. A. Moscarello, in preparation.
5 μmoles of 2-(N-morpholino)ethanesulfonate buffer, pH 5.7, 5 μl of 2% Triton X-100, and 25 μl of a sonicated enzyme suspension, in a total volume of 45 μl. For the measurement of activity toward desialyldegalactosylorosomucoid, the incubation medium was the same as above except for the replacement of GlcNAc by 1 mg of desialyldegalactosylorosomucoid (kindly supplied by Dr. H. Schachter, University of Toronto).

The assay mixtures were incubated for 2 hours at 37°, and the reaction was stopped by the addition of 5 μl of 2% sodium tetraborate, pH 0.2, containing 0.25 μM EDTA. Product formed was separated by high voltage electrophoresis and counted by liquid scintillation as in Schachter et al. (5).

The complete assay mixture contained 0.05 μmole of UDP-[3H]Gal, 5 μmoles of GlcNAc, 5 μmoles of MnCl₂, 5 μl of 5% Triton X-100, 5 μmoles of 2-(N-morpholino)ethanesulfonate (MES) buffer, pH 5.7, and 25 μl of enzyme suspension containing 0.03 mg of protein in a total volume of 45 μl. All assay mixtures were incubated for 2 hours at 37°.

Component omitted from complete assay mixture Complete activity

<table>
<thead>
<tr>
<th>Component omitted from complete assay mixture</th>
<th>Complete activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>5</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>24</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>62</td>
</tr>
<tr>
<td>MES buffer, pH 5.7</td>
<td>95</td>
</tr>
</tbody>
</table>

FIG. 1 (left). The initial rate was plotted as a function of increasing concentration of UDP-Gal. The assay contained Golgi membranes (0.03 mg), 0.05 μmole of UDP-[3H]Gal, 0.25 mg of GlcNAc, 5 μmoles of 2-(N-morpholino)ethanesulfonate buffer, pH 5.7, 5 ml of 5% Triton X-100, and 3 μmoles of MnCl₂ in a volume of 50 μl. After incubation for 3 min at 37°, the reaction was stopped with 5 μl of 2% sodium tetraborate containing 0.25 μM EDTA.

FIG. 2 (right). A, initial rate of transfer of UDP-[3H]Gal to desialyldegalactosylorosomucoid (Curve 1) and GlcNAc (Curve 2) as a function of concentration of acceptor. The assay contained Golgi membranes (0.03 mg), 0.05 μmole of UDP-[3H]Gal, varying concentrations of glycoprotein or GlcNAc, 5 μmoles of 2-(N-morpholino)ethanesulfonate buffer, pH 5.7, 5 μl of 5% Triton X-100, and 3 μmoles of MnCl₂ in a volume of 50 μl. The rate was determined as disintegrations per min transferred after incubation for 3 min at 37°. B, reciprocal plot 1/V X 1/S for glycoprotein; C, reciprocal plot 1/V X 1/S for GlcNAc.
Fig. 2A represents a plot of initial rate against acceptor sites for the two acceptors, GlcNAc (Curve 2) and desialyldegalactosylorosomucoid (Curve 1). When desialyldegalactosylorosomucoid was used as acceptor, the initial rate rose rapidly to a maximum at about 2 mM and fell sharply. With GlcNAc as acceptor, the initial rate was slower than with desialyldegalactosylorosomucoid and the maximum rate was not obtained until about 15 mM. Fig. 2, B and C, represents the reciprocal plots for the two acceptors, desialyldegalactosylorosomucoid and GlcNAc, respectively. The $K_m$ values were 0.7 mM for desialyldegalactosylorosomucoid and 3.9 mM for GlcNAc. The corresponding $V_{max}$ values were 370 and 600 dpm per min, respectively. Corresponding $K_m$ values for pork liver enzyme were 0.1, 4.6, and 1.0 for UDP-Gal, GlcNAc, and desialyldegalactosylorosomucoid, respectively (5).

**Table II**

**Inhibition of galactosyltransferase by puromycin**

Golgi membranes were preincubated with different concentrations of puromycin for 5 min at 37°C and then assayed to determine initial rate. The assay contained 0.05 μmole of UDP-[14C]Gal, 0.25 mg of GlcNAc, 5 μmoles of 2-(N-morpholino)ethanesulfonate buffer, pH 5.7, 5 μl of 5% Triton X-100, and 3 μmoles of MnCl₂. Two different Golgi suspensions were used, of protein concentrations 1.4 and 2.6 mg/ml, respectively.

<table>
<thead>
<tr>
<th>[Puromycin] in preincubation medium (mM)</th>
<th>Initial rate of galactosyltransferase (dpm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GlcNAc protein, 0.035 mg</td>
</tr>
<tr>
<td>0</td>
<td>582</td>
</tr>
<tr>
<td>1.24</td>
<td>425</td>
</tr>
<tr>
<td>1.84</td>
<td>280</td>
</tr>
<tr>
<td>2.45</td>
<td>145</td>
</tr>
<tr>
<td>3.06</td>
<td>50</td>
</tr>
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</table>

**Effect of Puromycin on Galactosyltransferase Activity in Vitro**

When Golgi membranes were preincubated with puromycin for 5 min at 37°C and subsequently assayed for galactosyltransferase activity using GlcNAc as acceptor, inhibition was observed. The degree of inhibition varied directly with the amount of Golgi protein and with the amount of puromycin (Table II). In the absence of puromycin the galactosyltransferase activity was 582 and 916 dpm per min for the two concentrations of Golgi used, 0.035 and 0.065 mg of protein per assay, respectively. Increasing the concentration of puromycin from 0 to 3.06 mM resulted in a progressive loss of activity so that at 3.06 mM the initial rate was only 8.6% of that of the uninhibited enzyme when the amount of protein was 0.035 mg. The effect was less marked at the higher concentration of Golgi protein, 0.065 mg. When the concentration of puromycin was 3.06 mM the initial rate was 31% of that of the uninhibited system for the higher concentration.

The effect of puromycin on galactosyltransferase was determined for the two substrates GlcNAc and desialyldegalactosylorosomucoid. Golgi membranes (0.035 mg of protein) were preincubated with puromycin for 5 min at 37°C. Initial rates were determined in the 3-min assay. The data are plotted as initial rate versus concentration of puromycin, and are shown in Fig. 3. Curve A shows the effect of puromycin on the initial rate when GlcNAc was used as acceptor; Curve B shows the effect when desialyldegalactosylorosomucoid was used.

When GlcNAc was used as acceptor (Curve A) only a small inhibition was observed up to a puromycin concentration of 1.5 mM. This was followed by a sharp fall in rate to less than 20% of the original rate when the puromycin concentration was 3 mM. From this concentration to 5.7 mM the initial rate remained constant at 6 to 7% of the original value. The inhibition observed when desialyldegalactosylorosomucoid was used as acceptor was different from that observed with GlcNAc. The binding of [3H]puromycin to Golgi membranes in vitro. Golgi membranes in water were incubated for various times with [3H]puromycin in the presence of various concentrations of puromycin. After various times the Golgi suspension was diluted to 0.5 ml with 2-(N-morpholino)ethanesulfonate buffer and filtered on a Millipore filter, washed 3 times with 0.5 ml of 2-(N-morpholino)ethanesulfonate buffer, pH 5.7, and the filter was counted.


**Table III**

**Effect of puromycin and related compounds on galactosyltransferase activity of rat liver Golgi**

Golgi membranes (0.035 mg) were preincubated for 5 min at 37°C in distilled water with the additions indicated and then assayed for galactosyltransferase activity. The assay medium contained 0.09 μmole of UDP-[3H]Gal, 0.23 mg of G1cNac, 5 μmoles of 2-(N-morpholino)ethanesulfonate buffer, pH 5.7, 5 μl of 5% Triton X-100, and 3 μmoles of MnCl₂ in 45-μl total volume. After 30-min incubation at 37°C the dpm transferred were measured as described in methods.

<table>
<thead>
<tr>
<th>Addition to the preincubation medium (μmole)</th>
<th>Galactosyltransferase activity (dpm/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5300</td>
</tr>
<tr>
<td>Puromycin (0.37)</td>
<td>70</td>
</tr>
<tr>
<td>Aminonucleoside (0.68)</td>
<td>6100</td>
</tr>
<tr>
<td>Aminonucleoside (1.37)</td>
<td>7000</td>
</tr>
<tr>
<td>Aminonucleoside (0.68) + phenylalanine (1.21)</td>
<td>6080</td>
</tr>
<tr>
<td>6-Dimethyladenosine (0.68)</td>
<td>7060</td>
</tr>
<tr>
<td>6-Dimethyladenosine (1.37)</td>
<td>6960</td>
</tr>
<tr>
<td>6-Dimethyladenosine (0.68) + phenylalanine (1.21)</td>
<td>7340</td>
</tr>
<tr>
<td>3'-Amino-3'-Deoxyadenosine (0.76)</td>
<td>4900</td>
</tr>
<tr>
<td>3'-Amino-3'-Deoxyadenosine (1.52)</td>
<td>4230</td>
</tr>
<tr>
<td>3'-Amino-3'-Deoxyadenosine (0.76) + phenylalanine (1.21)</td>
<td>3710</td>
</tr>
<tr>
<td>Ribose (1.34)</td>
<td>4970</td>
</tr>
<tr>
<td>Ribose (2.67)</td>
<td>4920</td>
</tr>
<tr>
<td>Phenylalanine (1.21)</td>
<td>8180</td>
</tr>
<tr>
<td>Phenylalanine (2.42)</td>
<td>7400</td>
</tr>
</tbody>
</table>

The effect of 200 μg (0.37 μmole) of puromycin was to decrease the rate from 5300 to 70 dpm/30 min, i.e. to 1.3% of the control activity. None of the other compounds reproduced the effect of puromycin. Some inhibition was observed with a combination of 200 μg each of 3'-amino-3'-deoxyadenosine and phenylalanine. The activity was decreased from 5300 to 3570 dpm/30 min or 67% of the control. Slight inhibition was observed with 3'-amino-3'-deoxyadenosine and with ribose. The effect of aminonucleoside, 6-dimethyladenosine, and phenylalanine separately appeared to be to increase the activity to 130 to 150% of the control values.

**DISCUSSION**

The action of puromycin at the ribosomal level has been shown to involve the release of nascent peptide chains by replacing aminoacyl-tRNA (17). Ultrastructural studies showed that puromycin causes disaggregation of polysomes in vitro and in vivo, but the effect was reversed rapidly (19).

In addition to its effect at the ribosomal level, puromycin has an effect on the assembly of sugars into glycoprotein. In the thyroid gland, puromycin completely inhibits the assembly of the oligosaccharide side chain (28). Sugars, such as mannose, near the peptide core were inhibited more than those farther away such as galactose (20).

The enzymes responsible for the sequential addition of sugars to the oligosaccharide chain are located in the Golgi apparatus. The addition of each sugar is accomplished by a specific glycosyltransferase. In this report, we have studied the effect of puromycin on galactosyltransferase of isolated Golgi fractions in vitro. We observed a direct effect of the drug on the enzyme activity, unrelated to the well known effect of puromycin on protein synthesis. The effect of puromycin was to cause a rapid inhibition of the enzyme activity when either N-acetlyglucosamine or desialydegalactosylsorosomucoid were used as acceptors of galactose. The inhibition was observed only when Golgi membranes were preincubated with puromycin. Addition of puromycin to the complete assay mixture after the addition of all components failed to elicit the inhibition. Studies with rat serum galactosyltransferase showed that puromycin was not an inhibitor of the soluble form of this enzyme (30).

A number of compounds structurally related to puromycin, such as the aminonucleoside portion (6-dimethylamino-9-[3'-amino-3'-deoxy-β-D-ribofuranosyl] pyrimidine), phenylalanine (the amino acid portion of puromycin, O-methoxyphenylalanine), 6-dimethyladenosine (the 3'-amino group of aminonucleoside was replaced by a hydroxyl group), 3'-amino 3'-deoxyadenosine (the 6-dimethyl group of aminonucleoside is missing), ribose (the sugar component of many nucleosides), and combinations of these compounds. Two concentrations of each compound were tested (200 and 400 μg). When combinations were added to the preincubation mixture, these were present in equal amounts. The data are shown in Table III.
Column chromatography of detergent-treated enzyme resulted in large losses of activity in the isolated fractions (1). The action of puromycin on the Golgi enzyme may result from the interaction of the molecule with some component of the membrane secondarily affecting the enzyme. An interaction with the membranes was demonstrated with radioactive puromycin which appeared to bind tightly. Further studies are underway to elucidate the mechanism of this interaction.

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Margaret Treloar, Jennifer M. Sturgess and Mario A. Moscarello


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