Transfer of mannosyl retinyl retinyl phosphate to protein

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ABSTRACT Upon incubation of [14C]mannose-labeled mannosyl retinyl retinyl phosphate with a membrane fraction from rat liver, mannosyl retinyl was transferred to an endogenous acceptor precipitable with chloroform/methanol to the extent of about 7%. The reaction proceeded linearly with time for 120 min at a pH optimum of about 7.0. The acceptor thus labeled with mannosyl retinyl could be solubilized by sodium dodecyl sulfate/mercaptoethanol. More than half of this acceptor appeared in the void volume of a Sephadex G-100 column. When it was digested with Pronase, a substantial proportion of it appeared between the void and bed volumes of a Sephadex G-100 column, thus indicating that it was a glycopeptide. In high-voltage paper electrophoresis, this glycopeptide moved to the cathode at low pH and to the anode at high pH. When digested with highly purified jack bean α-mannosidase, the glycopeptide released almost 50% of its radioactivity as mannosyl retinyl phosphate. That this transfer of mannosyl retinyl to glycoprotein from mannosyl retinyl phosphate does not take place via dolichyl mannosyl phosphate was shown by the fact that it is Mn2+ and Mg2+ independent, it is not inhibited by the presence of a 10-fold molar excess of nonradioactive GDP-mannose, and neither 14C-labeled dolichyl mannosyl phosphate nor 14C-labeled lipid pyrophosphoryl oligosaccharide could be detected during the incubation.

A metabolic function of vitamin A outside of the process of vision has been sought for many years. Our laboratory has established an involvement of vitamin A in glycoprotein synthesis (1–3). We have also shown that formation of a specific serum glycoprotein, α1-macroglobulin, presumably made in the liver, is vitamin A dependent (4). The biochemical mechanism whereby this action of the vitamin could be effected had remained obscure until the recognition that polysaccharides in the form of glycospholipids function as intermediates in the biosynthesis of complex glycans, in both bacterial and mammalian systems (5, 6).

Glycospholipids, consisting of a retinol moiety linked by a phosphodiester bond to mannosyl or galactosyl, have been formed from nucleotide sugars by mammalian enzyme preparations with retinol or retinyl phosphate (7–9) or have been found in oto (10, 11). We postulate that retinyl glycospholipids function in the transfer of sugar units in the synthesis of some, though not all, glycoproteins. At present it is not clear how this process relates to others involving dolichol intermediates (12). The present report, from which preliminary data have been published (13), describes the transfer of mannosyl retinyl phosphate (ManRet-P) to endogenous acceptors in rat liver membrane preparations.

MATERIALS AND METHODS

The preparation of vitamin A-deficient rats and the liver membrane fraction used in the incubations have been described (8). ManRet-P and dolichyl mannosyl phosphate (DolMan-P) were prepared from this membrane fraction by the use of GDP-[14C]mannose (New England Nuclear Corp., Boston, MA; 221 μCi/μmol) together with synthetic retinyl phosphate (8). All experiments involving retinol or ManRet-P were performed in dim incandescent light. We used differential solvent extraction, as described (8), to separate and purify ManRet-P and DolMan-P. In one large-scale run, we found the specific radioactivity of the pure ManRet-P thus prepared to be approximately 75 μCi/μmol, using the ultraviolet absorbitivity for retinol at the ultraviolet absorption maximum of 326 nm to measure the concentration of ManRet-P in the preparation.

After solvent extraction, amounts of ManRet-P or DolMan-P solution containing 10,000–30,000 dpm were freed of solvent in a stream of nitrogen and resuspended in 0.06 ml of 1.5% Zonyl A (from du Pont de Nemours & Co., Organic Chemicals Div., Wilmington, DE; previously adjusted to pH 7.0); to these solutions were added 0.3 ml of the liver membrane fraction (about 10 mg of protein per ml) in TKM buffer (0.25 M sucrose/50 mM Tris·Cl/25 mM KCl/3 mM MgCl2 at pH 7.6) (8). The solution was made 1 mM with respect to ATP and 10 mM with respect to MnCl2 (unless otherwise indicated in the tables), with a final volume of 0.6 ml, using 0.3 M Tris buffer (pH 7.0) to make up the volume. The presence of the TKM buffer made the final solution 2.5 mM with respect to Mg2+.

Incubation lasted 120 min (unless otherwise indicated in the figures) at 30°. The reaction was stopped by addition of 3 ml of chloroform/methanol (1:2, vol/vol), and the mixture was then allowed to stand 30 min at room temperature or overnight at 4°. The precipitate was collected by centrifugation, and the resulting pellet was extracted with 3 ml of chloroform/methanol (2:1). The pellet was then dried in a stream of nitrogen and dispersed in 2 ml of water by sonication. This suspension was then centrifuged, the aqueous solution was removed, and the pellet was resuspended in water. This process was repeated once. The resulting pellet was then extracted twice with 3 ml of chloroform/methanol/water (10:10:3, vol/vol). The final pellet was then subjected to one of two processes: it was either dissolved in 0.1 M Tris buffer (pH 6.8) containing sodium dodecyl sulfate (NaDodSO4) (2%) and mercaptoethanol (2.5%) by heating to 100° for 5 min (to be used for fractionation by gel filtration), or it was dissolved directly in 0.2 M NaOH for 1 hr at 60° (to be used for radioactivity determination in 0.5% 2,5-diphenyloxazole (PPO)/0.7% Bio-Solve BBS-3 liquid scintillation fluid and for protein determination by the method of Lowry et al. (14)). Wherever "cpm" are given in tables and figures, the efficiency of the liquid scintillation counter was 92%.

RESULTS AND DISCUSSION

Upon incubation of [14C]mannose-labeled ManRet-P with a membrane fraction from rat liver, mannosyl could be observed...
Protein 7.15 CHClJCH30H/H2O, 1:1:0.3

Water wash 1:2

ManRet-P. or little (10:10:3)-wash. DolMan-P, in known became and mannose aqueous This experiments.

radioactivity and DolMan-P. between the reaction was pH incorporation of mannose from ManRet-P and DolMan-P. Transfer from ManRet-P, as well as from DolMan-P, was linear with time of incubation up to at least 120 min. Since the specific radioactivity of the DolMan-P was not known, no comparison can be made between the percent incorporation of mannose from ManRet-P and DolMan-P. From Table 1, it is clear that about 60% of the radioactivity in ManRet-P is recoverable at the end of the reaction (for DolMan-P, this recovery was found to be 80%), as established by thin-layer chromatography. The water wash of the pellet contained about 30% of the incubated radioactivity. This aqueous fraction was shown to consist mostly of mannose and mannose phosphate. Very little of the label from ManRet-P became soluble in chloroform/methanol/water (10:10:3), a solvent known to extract lipid-oligosaccharide (15, 16). In contrast, in the reaction of our liver membrane fraction with DolMan-P, fully one quarter of the radioactivity transferred to total endogenous acceptors showed up in the chloroform/methanol/water (10:10:3) wash. This finding may indicate that little or no lipid-oligosaccharide intermediates are formed from ManRet-P.

The reaction was pH dependent and showed a broad maxi-

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity found in fractions, %</th>
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<tbody>
<tr>
<td>CHClJ/CH3OH, 1:2</td>
<td>64.0 ± 14.8</td>
</tr>
<tr>
<td>CHClJ/CH3OH, 2:1</td>
<td>5.0 ± 1.5</td>
</tr>
<tr>
<td>Water wash of protein pellet</td>
<td>32.7 ± 12.5</td>
</tr>
<tr>
<td>CHClJ/CH3OH/H2O, 1:1:0.3</td>
<td>1.13 ± 0.13</td>
</tr>
<tr>
<td>Protein</td>
<td>7.15 ± 1.5</td>
</tr>
</tbody>
</table>

* At the end of the incubation period, the protein was precipitated with chloroform/methanol (1:2), and percent uptake of radioactivity into the chloroform/methanol was determined. The same was done with other washings, as well as for the final protein pellet. Results shown are means and standard deviations from five separate experiments.

FIG. 1. Thin-layer chromatography of a sample of ManRet-P prepared by the method described (6), demonstrating that pure ManRet-P (free of substantial amounts of DolMan-P) can be obtained by the differential solvent extraction method.

Fig. 3. Fractionation of membrane acceptors from $^{14}$C-ManRet-P or Dol$^{14}$C-Man-P by gel filtration. \( \text{cpm} \), \( A \) at 280 nm. (A) $^{14}$C-ManRet-P was incubated and separated, and protein was dissolved in NaDodSO4/mercaptoethanol. The column (2.5 x 52.5 cm) of Sephadex G-25 (Pharmacia Fine Chemicals Co., Piscataway, NJ) was charged with 25,500 dpm (14.6 mg of protein) in 2.7 ml and eluted with 5 mM Tris buffer (pH 7.4). (B) Identical experiment, but incubation of Dol$^{14}$C-Man-P. The column (1.9 x 32 cm) was charged with 1776 dpm of membrane acceptor solution.

4B) was subjected to high-voltage electrophoresis on paper, a portion of it moved to the cathode at low pH and to the anode at high pH (Fig. 5), showing its zwitterionic nature. This finding showed it to be a glycopeptide, in which case at least part of peak I (Fig. 3A) would be a glycoprotein.

No attempt was made to analyze by Pronase digestion the product of transfer from DolMan-P to acceptor (Fig. 3B), to determine if glycopeptides obtained might be different from or similar to those from ManRet-P.

We next wanted to demonstrate that the sugar added from ManRet-P to the presumed glycoprotein was in fact mannose and proposed to subject the glycopeptide (peak III, Fig. 4B) to the action of highly purified jack bean $\alpha$-mannosidase (17). The standard procedure for such hydrolysis (18) is to incubate the glycopeptide with $\alpha$-mannosidase in 0.15 M citrate buffer (pH 4.5) at 37° for 48 hr. However, when we tested the mannosidase under such conditions, we found that 40% of the activity had been lost in 3 hr and 75% in 12 hr. Even when kept on ice, $\alpha$-mannosidase lost 50% activity in 6.5 hr.* Therefore, in order to demonstrate that $^{14}$C-mannose could be liberated from the glycopeptide, we incubated it with $\alpha$-mannosidase for 24 hr, with six separate additions of $\alpha$-mannosidase, as described in the legend to Fig. 6. During that time, nearly 50% of the radioactivity originally present as mannose was liberated from the glycopeptide, as mannose. We can conclude that: (a) $^{14}$C-ManRet-P transfers its sugar to glycoprotein as mannose; (b) at least 50% of the mannose transferred is in the $\alpha$ configuration; and (c) at least 50% of the mannose is in the end position of the sugar chains, perhaps as a result of incomplete glycosylation in our liver membrane fraction. It is also possible that the $\alpha$-mannosidase removed mannose moieties sequentially from a poly($\alpha$-mannose) chain.

Having thus proved that ManRet-P transfers its mannose to a glycoprotein of high molecular weight, we had to show that this transfer reaction is not the result of a formation of GDP-$^{14}$C-mannose by a reversal of the reaction by which ManRet-P is produced, a reaction catalyzed by the same membrane fraction as the transfer reaction (7):

\[
\text{retinyl phosphate + GDP-$^{14}$C-mannose} 
\rightarrow \text{$^{14}$C-ManRet-P + GDP.}
\]

The GDP-$^{14}$C-mannose thus formed could then react with dolichyl phosphate, which is normally present in the membrane fraction (12), and could thereby yield labeled DolMan-P. This DolMan-P could then transfer the labeled mannose via lipid oligosaccharide to protein. This sequence of reactions is improbable for the following reasons:

(a) It is unlikely that the membrane fraction would contain significant amounts of GDP.

(b) The transfer of mannose from ManRet-P to protein is manganese independent (Table 2), whereas the reaction of GDP-mannose with dolichyl phosphate would require man-

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* It has since been brought to our attention that the presence of Zn$^{2+}$ stabilizes $\alpha$-mannosidase (19).
ganase (12). Transfer was seen when both Mn²⁺ and Mg²⁺ were omitted from the incubation, and also when 10 mM EDTA was present.

(c) An approximately 10-fold molar excess of nonradioactive GDP-mannose, added to the reaction mixture with labeled ManRet-P to effect isotope dilution, showed no inhibition of the transfer of mannose to protein (Fig. 7).

(d) Incubation with [¹⁴C]mannose-1-P resulted in no significant labeling of protein.

(e) If transfer of mannose from ManRet-P occurred via formation of [¹⁴C]DolMan-P, the different labeling patterns seen in Fig. 2 would not be expected.

(f) No evidence was found for the presence of labeled DolMan-P or lipid-oligosaccharide. Incubations were stopped at 15 and 45 min, and the chloroform/methanol and chloroform/methanol/water extracts were applied to DEAE-cellulose columns. At both times, the only [¹⁴C]-labeled compounds found were eluted in the wash fraction and at very low concentrations of NH₄-acetate. These samples were analyzed by thin-layer chromatography in two systems, and only labeled mannose, mannose-P, and ManRet-P were present. Thus, at times of incubation when transfer to protein had occurred and was continuing, neither DolMan-P nor lipid-P-oligosaccharide could be detected.

We concluded, therefore, that this transfer was from Man-

Table 2. Effect of Mn²⁺ on [¹⁴C]mannose transfer from [¹⁴C]ManRet-P to protein

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Substrate</th>
<th>Addition of Mn²⁺, 10 mM</th>
<th>Protein, cpm</th>
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<tbody>
<tr>
<td></td>
<td>ManRet-P, cpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2,800</td>
<td>–</td>
<td>442</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>599</td>
</tr>
<tr>
<td>B</td>
<td>22,000</td>
<td>–</td>
<td>1580</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1650</td>
</tr>
</tbody>
</table>

FIG. 5. High-voltage paper electrophoresis of the glycopeptide obtained by proteolytic hydrolysis of [¹⁴C]glycoprotein (peak I, Fig. 3A). Peak III (Fig. 4B) (1051 dpm), representing the main glycopeptide peak, was divided into two equal portions and each was lyophilized. One was taken up in buffer at pH 1.9 (acetate/formic acid/water, 97:2.5:0.008) and the other was taken up in buffer at pH 10.6 (carbonate/bicarbonate); they were separately applied to Whatman no. 1 paper, which was subjected to electrophoresis, with unlabeled mannose and dinitrophenyllysine as markers. The pH 1.9 strip was subjected to 4000 V and 45-50 mA for 2 hr; the pH 10.6 strip underwent 3500 V and 85 mA for 2 hr. The papers were then dried and cut into 1-cm strips, which were placed in scintillation vials with 1 ml of water and allowed to stand for 1 hr. Radioactivity was determined in Triton X-100 scintillator.

FIG. 6. Paper chromatography of the mannose liberated by α-mannosidase from [¹⁴C]glycopeptide. The second portion (Fig. 5) of glycopeptide (peak III, Fig. 4B) (1051 dpm) was dissolved in 0.4 ml of 0.187 M sodium citrate buffer (pH 4.5). To this was added 1.77 units of α-1-mannosidase in 25 μl of acetate buffer (pH 6.0) at zero time, and after 3, 6, 9, 12, and 18 hr of incubation at 37°C. After 24 hr the sample was heated to 100°C for 5 min and centrifuged. The supernatant solution, combined with two methanol washes of the precipitate, was lyophilized; it was then dissolved in a small amount of water and chromatographed descendingly on Whatman 3MM paper (pyridine/n-butanol/0.1 M HCl, 3:5:2) for 42 hr. The paper was then dried, cut into 1-cm strips, and allowed to stand with 1.5 ml of water for 1 hr. Radioactivity was determined with 10 ml of Triton X-100/toluene/PPO scintillation fluid. (Upper) Radioactivity on the paper after a control incubation without enzyme; (Lower) release of mannose by the enzyme.

Ret-P directly to an endogenous acceptor, excluding intermediate conversion into DolMan-P. The experiments described thus far were all carried out with membrane fractions obtained from vitamin A-deficient rats. Identical results were obtained on repeating them with membranes from normal rats. With respect to Mn²⁺ independence of the reaction, we found, using liver membrane fractions from vitamin A-deficient and normal rats (three separate experiments for each), that on closer analysis, the formation of glycoprotein (peak I, Fig. 3A) remained independent of the presence or absence of 10 mM Mn²⁺ in the incubation medium, whereas the unknown peak II showed a Mn²⁺ dependence.

The question arises as to the nature of the protein that is
glycosylated by ManRet-P. Generally, the liver is regarded as a storage rather than a "target" organ of vitamin A. However, recent results from our laboratory (4) have shown that synthesis of a serum glycoprotein, α1-macroglobulin, presumed to be formed in the liver (20), is vitamin A dependent. Our present efforts are directed towards answering the question of whether or not this protein is an acceptor of mannose from ManRet-P.

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