MANNOSYLTRANSFER REACTIONS IN RABBIT LIVER MICROSOMES

J. S. Schutzbach and A. K. Verma

Department of Microbiology
and Institute of Dental Research
University of Alabama in Birmingham
Birmingham, Alabama 35294

Received May 11,1976

SUMMARY: Rabbit liver microsomes catalyzed mannosyltransfer from GDP-[14C]mannose to free D-mannose resulting in the synthesis of α-1,2-, α-1,3-, and α-1,6-mannosyl-mannose. Whereas formation of α-1,2-mannosyl-mannose was stimulated by the addition of manganese chloride or nickel chloride and was inhibited by EDTA, synthesis of α-1,3-mannosyl-mannose was unaffected by manganese or EDTA and was inhibited by nickel. Formation of α-1,6-mannosyl-mannose appeared to be stimulated by manganese and inhibited by nickel. These results suggest that three different mannosyltransferases were involved in the synthesis of mannosyl-mannose glycosidic linkages in rabbit liver.

A number of investigations in recent years have been concerned with the mechanisms by which mannose[1] is incorporated into mammalian glycoproteins (1-7). Most of these studies have demonstrated, either directly or indirectly that mannosyl-lipids are intermediates in the synthesis of the mannosyl-mannose glycosidic linkages. The mannosyl-lipids were shown to be similar to or identical with dolichol-monophosphate-mannose. In only one study, however, were the mannosyl-mannose linkages characterized as to both anomeric configuration and linkage position (7). An enzyme preparation from calf thyroids was found to transfer mannose from dolichol-monophosphate-mannose to α-methyl-mannoside and aryl-mannosides resulting in the formation of α-1,2-mannosyl-mannose linkages. By comparison, Levy et al. (8) have demonstrated mannosyltransfer directly from GDP-mannose to the exogenous acceptor N, N'-diacetylchitobiosyl-pyrophosphate-dolichol with the formation of a β-mannosyl-N-acetylglucosamine linkage. In this paper we present evidence showing that an enzyme preparation from rabbit liver can catalyze mannosyltransfer to exo-

---

1 All sugars are of the D-configuration.

genous mannose resulting in the formation of α-1,2-, α-1,3-, and α-1,6-mannosyl-mannose linkages. This is the first report on the synthesis of the α-1,3- and α-1,6-linked disaccharides by mammalian enzymes and our data suggest the possibility that there are at least three different enzymes catalyzing formation of mannosyl-mannose glycosidic linkages although final proof awaits separation and characterization of the enzymes involved.

Methods and Materials: The disaccharides, α-1,2-, α-1,3-, and α-1,6-mannosyl-mannose, and mannotriose containing mainly Man-α-1,2-Man-α-1,2-mannose and some Man-α-1,3-Man-α-1,2-mannose were prepared and characterized as previously described (9,10). All other chemicals and enzymes were purchased from commercial sources. Paper chromatography was carried out on Whatman No. 1 paper in the following solvent systems: (1) 1-propanol-ethyl acetate-water, 7:1:2; (2) ethyl acetate-pyridine-water, 8:2:1, and (3) ethyl acetate-acetic acid-water, 3:1:1. Electrophoresis was carried out at 30 volts per cm for 8 hrs in 0.04 M sodium borate, pH 9.2. Carbohydrates were detected with silver nitrate or with p-anisidine phthalate (11) and protein was determined by the procedure of Lowry et al. (12).

Preparation of Enzyme: Livers were obtained from 1.2-1.5 kg fasted New Zealand White rabbits and washed with 0.1 M Tris-acetate buffer, pH 7.3, containing 0.5 g EDTA and 0.5 g reduced glutathione per liter. All subsequent procedures were carried out at 0-4°. The liver was minced and 25 g were suspended in 25 ml of buffer containing 0.25 M sucrose. The mince was homogenized in a ground glass homogenizer and the homogenate was centrifuged at 10,000 x g for 15 min. The precipitate was discarded and the supernatant was centrifuged at 100,000 x g for 45 min. The pellets were pooled, resuspended in 3 volumes of buffer containing 0.25 M sucrose and again centrifuged at 100,000 x g for 45 min. The microsomal pellet was suspended in buffer to a protein concentration of 25-50 mg/ml.

Enzyme Assays: Assay I. Mannosyltransfer from GDP-mannose to exogenous mannose.

Reaction mixtures contained GDP-[^{14}C]mannose (0.1 μCi; 160-246 mCi/mmol, New England Nuclear), $MnCl_2$ (1 μmol), ATP (0.1 μmol), mannose (40 μmol), and enzyme (1.25-2.5 mg protein) in a total volume of 0.1 ml of buffer. After 60 min at 25° the reaction mixtures were applied to 4 x 10 cm strips of Whatman DE81 paper, and neutral products were eluted with water. The eluates were subjected to chromatography in Solvent 1 and the chromatograms were analyzed by autoradiography. Radioactive disaccharides were eluted with water and counted in a gas flow counter. Control reaction mixtures without added mannose were treated identically. The α-1,2- and α-1,3-linked disaccharides were then separated by electrophoresis in borate buffer, excised, and counted in toluene scintillation fluid. It has previously been shown that chromatography in solvent 1 and electrophoresis in borate buffer are sufficient to separate and identify all of the α-linked mannosbioses (9,10).

Assay II. Mannosyltransfer from GDP-mannose to endogenous lipid.

Reaction mixtures were prepared as described for Assay I. After 15 min at 25° the reaction mixtures were diluted by addition of 0.1 ml of water and heated at 100° for 3 min. To each reaction mixture was added 0.5 ml of

chloroform-methanol (2:1) and the solutions were thoroughly stirred on a vortex mixer. After centrifugation at 8,000 x g for 3 min the chloroform-methanol layers were transferred to a second tube. The aqueous phase and pellet at the organic phase-water interface were extracted twice with 0.25 ml chloroform-methanol and the organic layers were pooled with the first extract. Following extraction with 0.25 ml of Folch upper phase (13), the organic phase was transferred to a scintillation vial, taken to dryness at 60°, and counted in Triton-toluene scintillation fluid (14).

Incubation with α-Mannosidase: Samples of radioactive disaccharides (2100 cpm of α-1,6-mannosyl-mannose and 8100 cpm of a mixture of α-1,2- and α-1,3-mannosyl-mannose) were incubated with α-mannosidase (1 μg; Boehringer Mannheim) in 0.2 ml of 0.5 M ammonium acetate, pH 5.0, for 8 hrs at 25°. Reaction products were separated by chromatography in Solvent 1.

Results: Rabbit liver microsomes catalyzed mannosyltransfer from GDP-[^{14}C] mannose to free mannose resulting in the formation of products with the chromatographic mobilities of mannosyl-mannose disaccharides (Fig 1). Glycosyltransfer to free mannose did not occur when the GDP-mannose was replaced by any of the following compounds labeled with carbon-14 in the glycosyl-moiety: mannose-1-

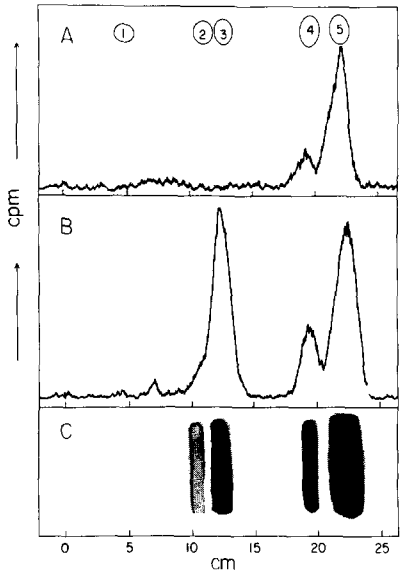

Fig. 1. Mannosyltransfer from GDP-[^{14}C]mannose to exogenous mannose. Reaction mixtures were as described for Assay I and the reaction products were separated by chromatography in Solvent 1. The figure shows radioactive strip scans of; (A) control reaction without mannose; (B) plus mannose. Full scale deflection equals 1000 cpm. In 1C is shown a tracing of the X-ray film after autoradiography. The standards are (1) mannotriose, (2) α-1,6-mannosyl-mannose, (3) α-1,2- and α-1,3-mannosyl-mannose, (4) glucose, and (5) mannose.

phosphate, UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, and GDP-fucose. Identification of Products: One of the radioactive products had the same mobility on paper chromatography as α-1,6-mannosyl-mannose (Fig 1) and was identified as such by the following analysis: a) complete acid hydrolysis in 1 N HCl for 2 hrs at 100° yielded only [^{14}C]mannose as determined by chromatography in Solvents 1, 2, and 3; b) reduction of the disaccharide with sodium borohydride, followed by complete acid hydrolysis liberated only [^{14}C]mannose and not mannitol as determined by chromatography in Solvent 2, indicating that the radioactive mannose was not at the reducing terminus; c) the radioactive product was completely degraded by α-mannosidase establishing that the linkage was of the α-configuration; and d) a sample of the disaccharide (4600 cpm) was mixed with unlabeled α-1,6-mannosyl-mannose and subjected to chromatography in solvent 1. As can be seen in Fig. 2, the radioactive product co-migrated with authentic standard.

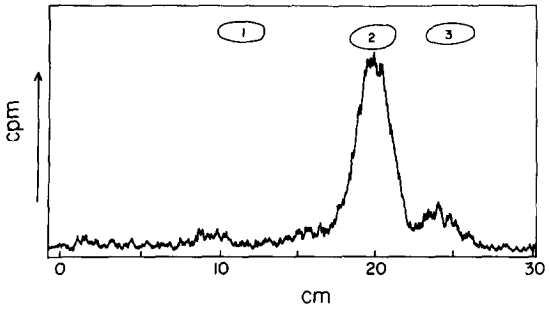

Fig. 2. Chromatography of α-1,6-[^{14}C]mannosyl-mannose. Carbon-14 labeled mannobiose was prepared as in Fig. 1B, and the α-1,6-mannobiose was eluted, mixed with authentic standard, and subjected to chromatography in solvent 1. The figure shows a radioactive strip scan of the chromatogram. Full scale deflection equals 300 cpm. The standards, are (1) mannotriose, (2) α-1,6-mannosyl-mannose and (3) α-1,2- and α-1,3-mannosyl-mannose.

As shown in Fig. 1, most of the radioactive material formed by mannosyl-transfer to mannose migrated faster than α-1,6-mannosyl-mannose in Solvent 1

and was found in the same position as α-1,2- and/or α-1,3-mannosyl-mannose. Since these two disaccharides could not be resolved by this procedure, further fractionation was carried out by electrophoresis in borate buffer. As is shown in Fig. 3A, approximately equal amounts of the 1,2- and the 1,3-linked disaccharides were present. When this material was analyzed as in a, b, and c above, all of the radioactivity was found as α-linked mannose in non-reducing terminii of the disaccharides.

Although α-1,4-mannosyl-mannose was not included as a reference in these procedures, electrophoresis in borate buffer has previously been shown to separate α-1,2-, α-1,3-, and α-1,4-mannosyl-mannose (9,10).

Mannosyltransfer to Other Acceptors: In addition to free mannose, the following saccharides were mannosyl-acceptors when tested in the standard assay at

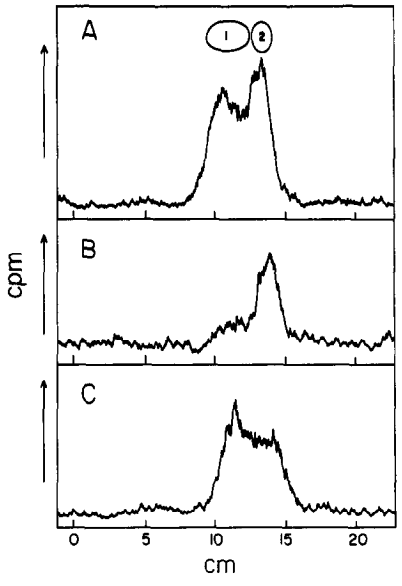

Fig. 3. Electrophoresis of [^{14}C]mannosyl-mannose. Carbon-14 labeled mannobiose was prepared in standard reaction mixtures as described in the text for Assay I (A) or prepared in similar reaction mixtures containing 10 mM EDTA (B) or 10 mM $NiCl_2$ (C) in place of $MnCl_2$. The α-1,2- and α-1,3-linked disaccharides were separated from α-1,6-mannosyl-mannose by chromatography in solvent 1 and were subjected to electrophoresis in 0.04 M sodium borate. The figure shows radioactive strip scans with full scale deflection equal to 300 cpm. The standards are (1) α-1,2-mannosyl-mannose and (2) α-1,3-mannosyl-mannose.

the concentrations indicated: 0.1 M α-methylmannoside (8330 cpm of product formed), 0.001 M p-nitrophenyl-α-mannoside (2890 cpm), and 0.04 M α-1,6-mannosyl-mannose (1395 cpm). The products formed were completely degraded by α-mannosidase but were not further characterized. The following compounds were not acceptors for mannosyltransfer when tested at the concentrations indicated: 0.2 M glucose, 0.2 M galactose, 0.2 M xylose, 0.04 M α-1,2-mannosyl-mannose, 0.04 M α-1,3-mannosyl-mannose, and 0.04 M sucrose.

Differentiation of Mannosyltransfer Reactions: Although all three of the α-linked mannobioses were synthesized under the conditions of the standard assay, it was possible to differentiate the reactions involved in the synthesis of each of the disaccharides. Thus the formation of α-1,2-mannosyl-mannose was stimulated over 3 fold by the addition of either $MnCl_2$ or $NiCl_2$ to the reaction mixture when compared to reactions containing 10 mM EDTA (Table I, Fig.3). In contrast, the synthesis of α-1,3-mannosyl-mannose was not stimulated by the addition of $MnCl_2$ and was inhibited by the addition of $NiCl_2$. The enzyme

TABLE I

Mannosyltransfer from GDP-[^{14}C]mannose to D-mannose and to Endogenous Lipid. Reactions were described under methods and materials except the divalent cation was as indicated or was replaced with EDTA.

Addition	Disaccharide Formed cpm/hr			Mannosyl-lipid cpm/15 min
(10 mM)	Man-2-Man	Man-3-Man	Man-6-Man	
$MnCl_2$	3350	2970	530	2840
$NiCl_2$	3360	690	100	1860
EDTA	1060	2610	240	120

catalyzing synthesis of α-1,6-mannosyl-mannose appeared to be stimulated by $MnCl_2$ and inhibited by $NiCl_2$.

Additional evidence that a separate reaction was involved in the synthesis of the α-1,6-linked disaccharide was provided when mannosyltransfer was assayed using microsomes prepared from the livers of adult rabbits (4-5 kg). Although the rates of formation of the α-1,2- and α-1,3-linked disaccharides were essentially unchanged, it was not possible to detect the formation of α-1,6-mannosyl-mannose.

Formation of Mannosyl-lipid: Concomitant with mannosyltransfer to exogenous acceptor, mannose was transferred from GDP-[^{14}C]mannose to endogenous lipid (Table I). The reaction had a requirement for divalent metals and was inhibited over 95% by 10 mM EDTA. The resulting product was soluble in chloroform-methanol (2:1) and was retained when passed through a column of DEAE-cellulose in chloroform-methanol. The [^{14}C]mannosyl-lipid was quantitatively eluted with 0.05 M ammonium acetate in chloroform-methanol. Hydrolysis of the mannosyl-lipid in 0.1 N HCl for 10 min at 100^{o} liberated all of the radioactivity as [^{14}C]mannose. These properties are consistent with those of a mannosyl-phosphate-lipid as has been described by a number of investigators (4,7,15).

Discussion: The presence of α-1,2- α-1,3-, and α-1,6-mannosyl-mannose linkages have been described in mammalian glycoproteins (16) but the enzymes involved in their synthesis have not been directly demonstrated. Adamany and Spiro have reported on an enzyme in calf thyroids that transfers mannose from dolichol-monophosphate-mannose to α-methylmannoside and aryl-mannosides with formation of α-1,2-mannosyl-mannose linkages (7). The thyroid enzyme did not utilize free mannose as an acceptor and did not synthesize 1,3- or 1,6-linkages. The results reported in this communication show that rabbit liver microsomes catalyze mannosyltransfer to mannose with the formation of all three mannosyl-mannose linkages and suggest that at least three different enzymes are involved.

It remains yet to be demonstrated that the liver and thyroid mannosyltransfer reactions are involved in glycoprotein biosynthesis, but such a function is implied for these enzymes. The results presented herein are also consistent with those showing that mannosyl-lipids are involved in the synthesis of some mannosyl-mannose glycosidic linkages (2-7). The formation of α-1,3-mannosyl-mannose, however, was optimal in the presence of 10 mM EDTA, whereas EDTA inhibited the formation of mannosyl-lipid over 95%. This suggests that the formation of the α-1,3-linkage occurred by mannosyltransfer directly from nucleotide sugar. A less likely explanation would be that a small pool of mannosyl-lipid turned over very rapidly and was involved only in the synthesis of the 1,3-linkage since formation of the other disaccharides was inhibited by EDTA. Complete characterization of the reactions involved in the synthesis of the mannosyl-mannose linkages, however, must await solubilization and purification of the individual mannosyltransferases.

ACKNOWLEDGMENTS: This investigation was supported by NIH research grants CA 16777 from the National Cancer Institute and DE-2670 from the National Institute of Dental Research and in part by a Faculty Research Grant of the University of Alabama in Birmingham, and by an Institutional Grant by the American Cancer Society. The expert technical assistance of Ray Sitz is gratefully acknowledged.

REFERENCES

1. Caccam, J.F., Jackson, J.J. and Eylar, E.H. (1969) Biochem. Biophys. Res. Commun. 35, 505-511.
2. Richards, J.B. and Hemming, F.W. (1972) Biochem. J. 130, 77-93.
3. Behrens, N.H., Carminatti, H., Staneloni, R.J., Leloir, L.F. and Cantarella, A.I. (1973) Proc. Nat. Acad. Sci. USA 70, 3390-3394.
4. Baynes, J.W., Hsu, A.F. and Heath, E.C. (1973) J. Biol. Chem. 248, 5693-5704.
5. Martin, H.G. and Thorne, K.J.I. (1974) Biochem. J. 138, 281-289.
6. Adamany, A.M. and Spiro, R.G. (1975) J. Biol. Chem. 250, 2830-2841.
7. Adamany, A.M. and Spiro, R.G. (1975) J. Biol. Chem. 250, 2842-2854.
8. Levy, J.A., Carminatti, H., Cantarella, A.I., Behrens, N.H., Leloir, L.F., and Tabora, E., (1974) Biochem. Biophys. Res. Commun. 60, 118-125.
9. Ankel, H., Ankel, E., Schutzbach, J.S. and Garancis, J.C. (1970) J. Biol. Chem. 245, 3945-3955.

10. Schutzbach, J.S. and Ankel, H. (1971) J. Biol. Chem. 246, 2187-2194.
11. Zweig. G. and Whitaker, J.R. (1967) Paper Chromatography and Electrophoresis, V. 1 pp 232-266, Academic Press, New York.
12. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Folch, J., Lees, M. and Stanley, H.S. (1957) J. Biol. Chem. 226, 497-509.
14. Patterson, M.S. and Green, R.C. (1965) Anal. Chem. 37, 854-857.
15. Wedgwood, J.F., Strominger, J.L. and Warren, C.D. (1974) J. Biol. Chem. 249, 6316-6324.
16. Baenziger, J. and Kornfeld, S. (1974) J. Biol. Chem. 249, 7270-7281.