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MEMBRANE-BOUND MANNOSYL TRANSFERASE IN YEAST GLYCOPROTEIN BIOSYNTHESIS

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SUMMARY

A membrane-bound enzyme from *Saccharomyces cerevisiae* catalyzes the transfer of mannose from GDP-mannose to mannose, mannobiose and mannotriose giving rise to the corresponding higher homologues. The transfer is highly specific concerning the mannosyl donor as well as the acceptor. The reaction leading to mannobiose has been studied in more detail. The formation of mannobiose is absolutely dependent on Mn^{2+} and the pH optimum is close to pH 8. The K_m values for GDP-mannose and mannose are 0.25 and 100 mM respectively. The linkage in mannobiose was shown to be $\alpha(1\rightarrow2)$ and the newly attached mannosyl moiety appeared in the nonreducing terminus of the molecule.

The same enzyme fraction incorporates radioactivity from GDP-[^{14}C]mannose into endogenous glycoproteins as well as into exogenous HCl-treated mannan glycoprotein. In each case most of the radioactivity can be released by β-elimination. Evidence is presented that no lipid intermediate is taking part in mannosyl transfer reactions to mannose or mannosyl groups as acceptor in contrast to the mannosyl transfer directly to serine and threonine of the glycoprotein.

INTRODUCTION

The major components of the yeast cell wall are mannan, glucan and protein; much of this material is in the form of glycoprotein [1]. In previous studies from this laboratory it has been demonstrated, that a particulate fraction from *Saccharomyces cerevisiae* catalyzes the transfer of the mannosyl residue from GDP-mannose to exogenous as well as endogenous dolichol monosphosphate (DMP) [2, 3]. DMP-mannose has been suggested to be a "lipid" intermediate in the biosynthesis of mannan glycoprotein. In subsequent work evidence has been presented, however, that only those mannosyl residues immediately linked to protein are incorporated via DMP, while additional mannose units are attached directly by GDP-mannose [4]. Furthermore the glycoprotein fraction synthesized from GDP-mannose as well as from DMP-mannose seemed to be rather heterogenous.

Abbreviation: DMP, dolichol monophosphate.

In this communication it will be shown that the same particulate preparation, responsible for the mannosylation of glycoprotein when endogenous acceptor is used, can transfer the mannosyl moiety from GDP-mannose but not from DMP-mannose specifically to mannose or oligomannoses. Also HCl-treated high molecular mannan glycoprotein works as exogenous acceptor. These transfer reactions simulate the transfer of mannosyl groups from GDP-mannose to mannosyl residues of endogenous glycoproteins.

MATERIALS AND METHODS

Enzyme preparation

S. cerevisiae (strain 66.24, Fleischmann Laboratories) was grown in the medium of Ghosh et al. [5] and harvested in the middle of the log phase. The cells were washed one time with 50 mM Tris–HCl (pH 7.4), containing 0.15 mM $MgCl_2$ and 5 mM mercaptoethanol and broken in a Bio-X-press. After centrifugation at 600 × *g* for 5 min to remove unbroken cells, the supernatant was spun at 48 000 × *g* for 20 min; the pellet was washed by resuspending it in the same medium, centrifuged again and suspended in 50 mM Tris–HCl (pH 7.4) containing 3.5 mM $MgCl_2$ and 5 mM mercaptoethanol.

Assay procedures

Test I. To measure the mannosyl transfer from GDP-[^{14}C]mannose to exogenous acceptors, endogenous glycoprotein and to endogenous DMP the following mixture has been used: 5 mM Tris–HCl (pH 7.4), 7 mM $MgCl_2$, 5 mM $MnCl_2$, 0.2 mM GDP-[^{14}C]mannose (spec. act. 8 Ci/mole), 20 mM mannose, unless otherwise indicated, and 0.1–0.2 mg protein (particulate fraction) were incubated in a total volume of 70 μl at 21 °C. The reaction mixture was stopped with 0.3 ml ethanol and separated on Whatmann paper No. 1 in solvent system A. Radioactive spots were located with a strip scanner, cut out and measured directly on paper in a scintillation counter in toluene-2,5-diphenyloxazol. If the incorporation into DMP or only into glycoprotein material was tested, the reaction was stopped with 1 ml chloroform–methanol (3:2, v/v) and treated further according to Behrens and Leloir [6].

Test II. This assay was used to measure mannosyl transfer from various possible donors to [^{14}C]mannose. 5 mM Tris–HCl (pH 7.4), 7 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM donor, 0.25 mM [^{14}C]mannose (spec. act. 35 Ci/mole) and 0.1–0.2 mg protein (particulate fraction) were incubated in a total volume of 70 μl at 21 °C for 3 h. The reaction was terminated with 0.3 ml ethanol and radioactivity detected according to Test I.

Test III. Mannosyl transfer from DMP-[^{14}C]mannose, prepared as previously described [4], has been tested in the following way. 50 000 cpm of DMP-[^{14}C]mannose, 5 mM Tris–HCl (pH 7.4), 0.08 % Triton X-100, 7 mM $MgCl_2$, 5 mM $MnCl_2$, exogenous acceptors as indicated and 0.1–0.2 mg protein (particulate fraction) were incubated in a total volume of 70 μl at 21 °C. The reaction was stopped with 0.3 ml ethanol and monitored as described above.

Preparation of mannosyl acceptors

Bakers yeast mannan was isolated according to Cifonelli and Smith [7] and submitted to acetolysis following the procedure of Kocourek and Ballou [8], using a mixture of acetic anhydride–acetic acid–H_2SO_4 (10:10:1, by vol.). The deacetylated acetolysis products were applied to a column of Sephadex G-25, superfine, and eluted with water. Three main fractions were collected: Fraction M(mannose), Fraction M_2 (*O*-α-D-manosyl-(1→2)-mannose), Fraction M_3 (mixture of *O*-α-D-mannosyl-(1→2)-mannosyl-(1→2)-mannose and *O*-α-D-mannosyl-(1→3)-mannosyl-(1→2)-mannose). The elution pattern was checked by thin-layer chromatography in Solvent C (developed three times). The pooled fractions were purified further by paper chromatography on Whatmann paper No. 3 in Solvent A. *O*-α-D-mannosyl-(1→6)-mannose was prepared by acid reversion according to Jones and Nicholson [9] and purified by paper chromatography in Solvent B, *O*-β-D-mannosyl-(1→4)-mannose was a gift from Dr Whistler.

Yeast cell wall mannan glycoprotein (Fraction A) was prepared by extraction of yeast cell walls with ethylenediamine as described by Sentandreu and Northcote [10]. Hydrolysis was carried out with 1 M HCl at 100 °C for 1–4 h, the solution was then neutralized, dialyzed against water and lyophylisized. The hydrolyzed fraction is called Fraction A-HCl.

$NaBH_4$ reduction and α-mannosidase treatment of [^{14}C]mannobiose

To 50 μl aqueous sample of radioactive Fraction M_2, 100 μmoles $NaBH_4$ were added and allowed to stand at room temperature overnight. The solution was deionized with Dowex 50 (H^+) resin and repeatedly evaporated in the presence of methanol to remove boric acid. Hydrolysis was carried out with 1 M HCl in sealed tubes at 100 °C for 2 h.

100 μl radioactive Fraction M_2, dissolved in 50 mM sodium citrate (pH 4.5) was incubated with 1 mg of α-mannosidase, hemicellulase (β-mannosidase), or emulsin (α-mannosidase) overnight at room temperature. The whole mixture was then stripped on a chromatogramm and developed in Solvent A. Jack bean meal has been used as source for α-mannosidase. The enzyme has been partially purified (gel filtration fraction) according to Yu-Teh Li et al. [11].

Chromatography and electrophoresis

Whatmann paper No. 1 was used for paper chromatography and Silica gel G plates from Merck for thin-layer chromatography. The following solvent systems have been used: (A) *n*-butanol–ethylacetate–acetic acid–water (40:30:25:40, by vol.); (B) *n*-propanol–ethylacetate–water (70:10:20, by vol.); (C) ethylacetate–pyridine–water (5:3:2, by vol.).

Electrophoresis was carried out on Whatmann paper No. 1 at 30 V/cm for 8 h in 0.04 M sodium borate (pH 9.2).

Sugars were detected on paper chromatograms with alkaline silver nitrate [12] and on thin-layer plates by charring with H_2SO_4.

RESULTS

Mannosyl transfer to mannose and oligomannoses

Transfer reaction to mannose. Incubation of the particulate fraction with GDP-[^{14}C]mannose and mannose produced a labelled compound with chromato-

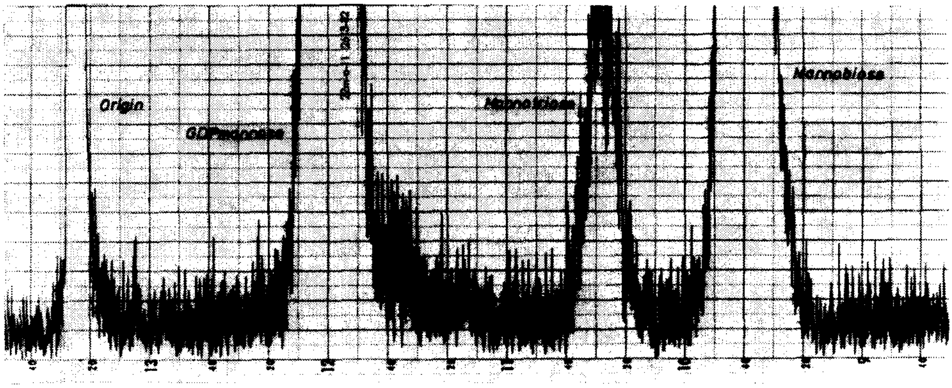

Fig. 1. Chromatographic separation of the products obtained in an incubation experiment with GDP-[^{14}C]mannose, mannose and particles according Test I.

graphic properties characteristic of mannobiose, Fraction M_2 (Fig. 1). When the amount of radioactivity in Fraction M_2 was high also a second compound appeared, which migrated like mannotriose, Fraction M_3. It arises via newly synthesized Fraction M_2. In the absence of mannose no radioactivity appeared in Fractions M_2 and M_3. In addition to these compounds an incorporation of radioactivity into material remaining at the origin always has been observed. This represents mannosylation of glycoproteins present as acceptor in the membrane fraction [4]. The incorporation of mannose from GDP-mannose into Fraction M_2 is linear with time and protein concentration (Fig. 2).

Characterization of the ^{14}C-labelled product. The configuration of the newly formed glycosidic linkage was determined with specific mannosidases. Incubation of the isolated Fraction M_2 product with jack bean α-mannosidase or emulsin released radioactivity as free mannose shown by chromatography in Solvent A. In

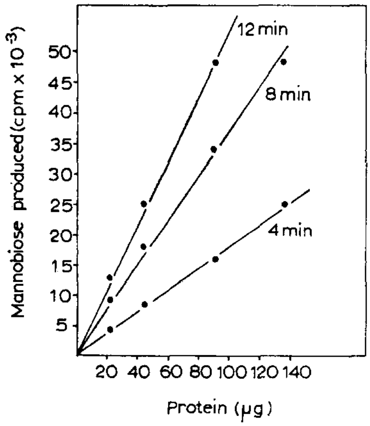

Fig. 2. Mannosyl transfer from GDP-[^{14}C]mannose to mannose as a function of time and protein concentration. The procedure was carried out according Test I with 10 mM mannose acceptor concentration.

contrast Fraction M_2 was resistant to hemicellulase (β-mannosidase) under the same reaction conditions. The linkage, therefore, is an α one. According to Schutzbach and Ankel [13] the possible linkages of mannobiose can be identified by chromatographic and electrophoretic methods. Fraction M_2 had the same chromatographic mobility in Solvent B and the same electrophoretic mobility in borate buffer as authentic *O*-α-D-mannosyl-(1→2)-mannose, which was prepared by acetolysis of yeast mannan [8]. Furthermore a sample of unlabelled Fraction M_2, synthesized enzymatically on a larger scale but otherwise identical conditions (only the concentration of GDP-mannose and mannose were higher than in the standard incubation) was kindly identified by Drs Lindberg and Lönngren with gas–liquid chromatography and mass spectrometry. Besides a 1→2 linkage also a 1→6 linkage has been found in this sample (ratio $\approx$ 2:1). Cochromatography of this sample with the radioactive compound revealed that the latter one consists only (i.e. more than 90%) of Fraction M_2 with 1→2 linkage. From this analysis we conclude the structure to be *O*-α-D-mannosyl-(1→2)-mannose.

The [^{14}C]mannobiose has been reduced by $NaBH_4$ and then hydrolyzed with 1 M HCl. Radioactivity has been found only in mannose, indicating that the mannosyl moiety of GDP-[^{14}C]mannose is transferred only to the nonreducing end of mannose.

Localisation of the mannosyl transferase. If the enzyme responsible for the synthesis of Fraction M_2 is indeed involved in the synthesis of membrane bound glycoproteins, one would expect that it were also insoluble. As can be seen in Table I, most of its activity is found in the pellet obtained after centrifugation at 100 000 × *g*. Thus it behaves like the enzyme responsible for the synthesis of mannose containing glycoproteins.

TABLE I

LOCALISATION OF THE ACTIVITY FOR FRACTION M_2 SYNTHESIS AND MANNAN GLYCOPROTEIN SYNTHESIS IN THE PARTICULATE FRACTION

Cells were harvested and broken in the usual way as described in Methods. After centrifugation at 600 × *g* for 5 min the supernatant was further centrifuged at 100 000 × *g* for 60 min. The pellet was dissolved in 50 mM Tris–HCl (pH 7.4) containing 3.5 mM $MgCl_2$ and 5 mM mercaptoethanol. Suitable aliquots of supernatant 600 × *g*, of supernatant 100 000 × *g* and of pellet 100 000 × *g* were tested by Test I for activity. Protein determination was carried out according to Lowry et al. [14].

Fraction	Glycoprotein synthesis		Fraction M_2 synthesis	
	Total activity (cpm × 10^{-3} per min)	Spec. act. (cpm × 10^{-3} per min per mg)	Total activity (cpm × 10^{-3} per min)	Spec. act. (cpm × 10^{-3} per min per mg)
1. Supernatant 600 × *g*	1500	4 930	1455	4 800
2, Supernatant 100 000 × *g*	216	1 215	470	2 640
3. Pellet 100 000 × *g*	1180	15 960	1178	15 920

Metal ion requirement. Mn^{2+} is essential for the transfer reaction and cannot be replaced by Mg^{2+}. As can be seen in Fig. 3 maximal activation is achieved at a concentration of about 5 mM. Is Mn^{2+} and Mg^{2+} simultaneously present the acti-

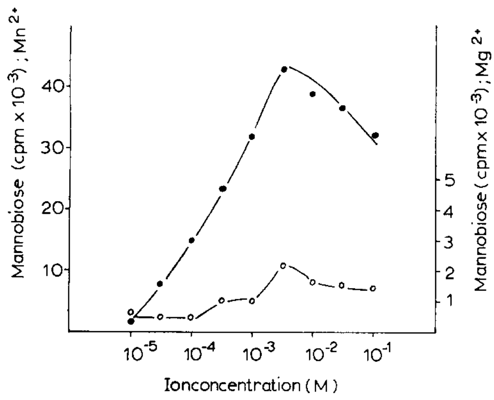

Fig. 3. Mannosyl transfer from GDP-[^{14}C]mannose to mannose as a function of Mn^{2+} and Mg^{2+} concentration. Reaction was carried out with Test I, except that the ion concentration was varied. ●—●, Mn^{2+}; ○—○, Mg^{2+}.

vation is more than only additive. Taking the reaction rate in the presence of 1 mM Mn^{2+} plus 1 mM Mg^{2+} as 100 the following relative rates have been observed: Mn^{2+}, 83; Mg^{2+}, 4; Al^{3+}, 9; Zn^{2+}, 6; Fe^{3+}, 3; Ni^{2+}, 3; Ca^{2+}, 3 (all cations at 1 mM).

Effect of pH. The pH dependence of the rate of mannobiose synthesis is illustrated in Fig. 4. The reaction showed an optimum around pH 8. Tris–HCl stimulates the reaction in comparison to Tris–maleate buffer.

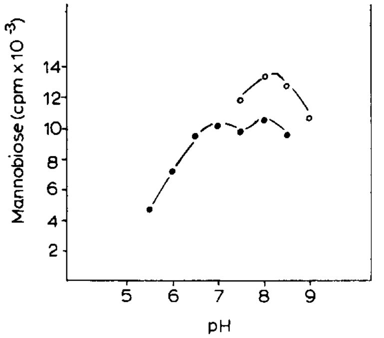

Fig. 4. Mannosyl transfer from GDP-[^{14}C]mannose to mannose as a function of pH. Test I was used with a final buffer concentration of 0.1 M. ●—●, Tris–HCl; ○—○, Tris–maleate.

Enzyme Kinetics. K_m values for mannose and GDP-mannose have been determined (Fig. 5). The Michaelis constant for mannose was found to be 50 mM in the presence of 0.2 mM GDP-mannose. A K_m value of 0.25 mM has been obtained for GDP-mannose in the presence of 100 mM mannose.

Donor specificity. The mannosyl transfer to mannose is highly substrate specific (Table II). When GDP-mannose was replaced by ADP-mannose, UDP-mannose or mannose phosphates, no transfer could be observed. Likewise other sugar nucleotide compounds e.g. GDP-glucose or UDP-galactose are completely inactive.

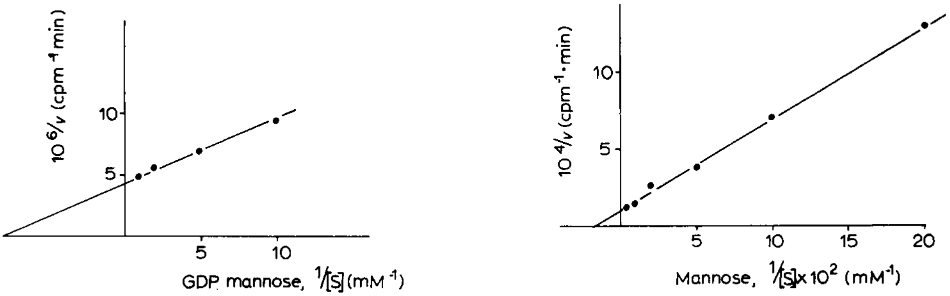

Fig. 5. Mannosyl transfer from GDP-[^{14}C]mannose to mannose as a function of substrate concentration. Lineweaver–Burk plots for GDP-mannose and mannose. Test I was used as described.

TABLE II

DONOR SPECIFICITY OF MANNOSYL TRANSFER TO MANNOSE

The donorspecificity was tested by measuring mannosyl transfer to [^{14}C]mannose with Test II. Incubation time was 3 h.

Donor	Reaction product (cpm)		
	Fraction M_2	Fraction M_3	Fraction M_4
GDP-mannose	34 765	8014	4100
ADP-mannose, UDP-mannose, mannose-1-*P*, mannose-6-*P* man-α-ONp* and mannose	do not act as donors		

* *p*-Nitrophenyl-α-D-mannopyranoside.

Acceptor specificity. The particulate system transfers the mannosyl moiety from GDP-mannose not only to mannose, but also to Fractions M_2 and M_3 (even with a 4- and 3-fold higher rate, respectively), thus giving rise to the corresponding higher homologues (Table III). Fraction M_2 is the best substrate besides methyl-α-D-mannoside. For activity it seems that the C-1 and C-6 position of the molecule must

TABLE III

ACCEPTOR SPECIFICITY OF MANNOSYL TRANSFER FROM GDP-MANNOSE

Acceptor specificity was determined with Test I. Final concentration of the various acceptors was 20 mM. Incubation time was 10 min.

Acceptor	Reaction product (cpm)
Fraction M	14 300 (Fraction M_2)
Fraction M_2	54 714 (Fraction M_3)
Fraction M_3	43 870 (Fraction M_4)
Methyl-α-D-mannoside	82 940 (Fraction M_2-CH_3)
Mannose-1-*P*	0
Mannose-6-*P*	0
4-*O*-β-M_2 fraction	0

Mannitol, *N*-acetylglucosamine, *N*-acetylmannosamin, inositol, glucose arabinose, xylose, galactose do not act as acceptors.

not be phosphorylated. Furthermore the α-configuration (see results with the 4-*O*-β-M_2 fraction) as well as probably the pyranose structure (see results with mannitol) are important. No transfer took place to *N*-acetylglucosamine, glucose or galactose.

Mannosyl transfer to high molecular acceptors

The particulate fraction has mannosyl transfer activity towards endogenous acceptors, which have been shown to be glycoproteins [4]. It was of interest, therefore, to see whether also mannose can be attached to exogenous polymeric acceptors. Mannan glycoprotein (Fraction A) was isolated from yeast according to Sentandreu and Northcote [10] and added to the incubation mixture. Only a small, but significant stimulation occurred (Table IV). Removal of mannoses, with 1 M HCl for 2 h, however, resulted in about a 4-fold increase in respect to the endogenous synthesis. A further treatment of Fraction A with HCl caused a complete loss of the stimulatory effect. This indicates, that there has to exist a specific sugar arrangement on the acceptor. The amount of mannose incorporated is linear with time and with the concentration of Fraction A-HCl (Fig. 6). About 70% of the radioactivity found in the exogenous acceptor can be cleaved by 0.1 M NaOH in 24 h at room temperature; this is the same amount as has been observed for the endogenous acceptor. The mild alkaline treatment is considered to be specific for breaking O-glycosidic linkages to serine or threonine [15]. Glucomannan (Fraction B) [10] or mannan without the protein component, both yeast cell wall components, do not work as acceptors.

TABLE IV

TRANSFER FROM GDP-MANNOSE AND DMP-MANNOSE TO VARIOUS ACCEPTORS

Transfer from DMP-mannose (30 min incubation time) and GDP-mannose (15 min incubation time) were carried out as described in Methods. The acceptor concentration was for mannose 10 mM, for Fraction A, Fraction A-HCl and mannan 0.5 mg, for mannoglucan 0.2 mg.

Acceptor	Transfer from GDP-mannose radioactivity (cpm) at the origin	Transfer from DMP-mannose radioactivity (cpm)	
		At the origin	In oligomannosides
Only endogenous acceptor present	11 776	5773	0
Fraction A	14 027	5275	–
Fraction A-HCl, 2 h	38 375	6169	–
Fraction A-HCl, 4 h	11 534	5708	–
Mannan (no protein portion present)	10 054	–	–
Mannoglucan	11 230	–	–
Mannose	11 252	6374	0

The question of the participation of a "lipid" intermediate

The question arises, whether mannose-linked "lipid" is contributing in the mannosyl transfer reactions from GDP-mannose to the various acceptors. Such a "lipid" participation has been shown for specific mannosyl linkages when endogenous glycoprotein is mannosylated [3, 4]. Two observations speak against an involvement

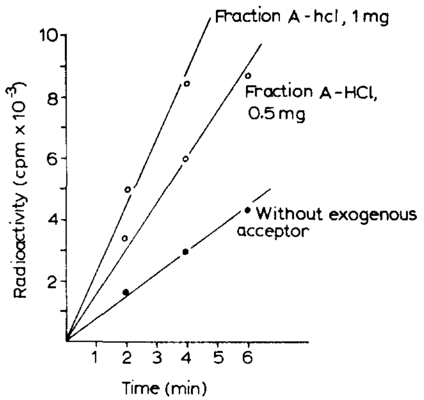

Fig. 6. Mannosyl transfer from GDP-[^{14}C]mannose to Fraction A-HCl. Fraction A was isolated and treated with 1 M HCl for 2 h as described in Materials and Methods.

of DMP in the reactions described here. As shown in Table IV no additional radioactivity has been found at the origin when DMP-[^{14}C]mannose was incubated as mannosyl donor and Fraction A-HCl as acceptor. With DMP-[^{14}C]mannose as donor and mannose as acceptor no radioactivity in Fraction M_2 or other oligomannoses has been observed. There was only an incorporation into the endogenous acceptor in all cases. The same particulate fraction, however, transferred mannose directly from GDP-mannose, as discussed above. In Table V Fraction M_2 synthesis is compared to the incorporation of [^{14}C]mannosyl residue into the lipid fraction depending on the age of the particulate fraction stored at 4 °C. While the transfer to mannose is not influenced within seven days a drastic decrease occurs in the incorporation of radioactivity into the endogenous lipid. This result likewise is not in agreement with a possible participation of a lipid intermediate.

TABLE V

MANNOSYL TRANSFER FROM GDP-MANNOSE TO MANNOSE AND ENDOGENOUS LIPID FRACTION, DEPENDING ON THE PARTICLE AGE

The transfer was measured in parallel incubation mixtures with Test I, except that 100 mM mannose was used. Incubation time was 2 min. One sample was stopped with 0.3 ml ethanol, chromatographed and checked for Fraction M_2; the other sample was terminated with 1 ml chloroform–methanol (3:2, v/v) and checked for lipid.

Fraction	Radioactivity (cpm) incorporated	
	Into Fraction M_2	Into endogenous lipid
Particle, fresh	12 661	1268
Particle, 7 days	13 534	200

DISCUSSION

Yeast cell wall contains a number of glycoproteins such as mannan glycoprotein, invertase and acidic phosphatase [16]. Little is known about the biosynthesis of yeast glycoproteins. It has only been shown that in the case of mannan glycoprotein the synthesis of the protein portion has to occur for mannose incorporation to take place in vivo [17]. Farcas et al. [18] have demonstrated that secretion of glycoproteins by yeast protoplasts can be inhibited by 2-deoxyglucose.

For animal systems it has been shown that highly specific membrane-bound glycosyltransferases are responsible for the stepwise addition of sugars to the protein moiety [19]. In several cases the involvement of "lipid intermediates" especially of DMP in the glyosylation of glycoproteins has also been suggested [20–23]. For *S. cerevisiae* evidence has recently been presented [4] that DMP transfers mannosyl groups preferentially to those positions of oligomannose side chains, which are linked to serine and/or threonine of the protein. The addition of further mannose molecules to that first one is thought to proceed directly from GDP-mannose as donor.

The results reported in this paper strongly support this view. From the particulate fraction of yeast cells a new enzyme is described, which transfers mannose from GDP-mannose to mannose, mannobiose or mannotriose. The behaviour of this enzyme parallels in all respect the activity of the membranes to mannosylate endogenous proteins. It has not been possible to obtain any evidence for the participation of a "lipid intermediate" in the formation of the soluble oligomannose units. The same has been true for the stimulated incorporation of radioactivity into the alcohol insoluble material, when Fraction A (the mannan glycoprotein of Sentandreu and Northcote [10]) has been added. The observation on the other hand, that Fraction A treated 2 h with HCl was most active in stimulating incorporation into β-eliminable positions but was inactive after a 4 h acid treatment, suggests that a partial but not total hydrolysis of the oligomannose side chains of the protein produces optimum acceptor qualities. It seems not possible, however, to add the first mannosyl residue, which is transferred via DMP, to exogenous acceptor molecules. That the stimulated incorporation of radioactivity is indeed due to the mannosylation of the protein portion of Fraction A can be seen from the fact that most of the [^{14}C]mannose incorporated is β-eliminable and that the addition of mannan itself does not result in any stimulation. Schutzbach and Ankel [13] have described a quite similar mannosyl transferase activity from *Cryptococcus laurentii.* No data have been obtained, however, to show that this enzyme could be involved in the mannosylation of glycoproteins.

In summary, therefore, the reactions described are thought to participate in the biosynthesis of oligomannose side chains of yeast glycoproteins. Such oligomannose units have been shown to occur serine and threonine bound in the mannan glycoprotein of yeast cell walls [24] but are also present in other yeast glycoproteins [25, 4]. The sequential addition of these mannose units, except the very first one, thus takes place directly via GDP-mannose.

ACKNOWLEDGEMENT

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