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EFFECT OF DIVALENT METAL IONS ON THE SYNTHESIS OF OLIGOSACCHARIDE SIDE CHAINS OF *NEUROSPORA CRASSA* GLYCOPROTEINS

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Abstract—*Neurospora crassa* membrane preparations incorporated mannose from GDP-mannose-[^{14}C] in the presence of Mg^{2+} into a polyprenol lipid and side chains of protein acceptor(s), which are labile on hydrolysis in weak base. The addition of Mn^{2+} to the reaction mixtures does not affect mannosyl lipid synthesis but it stimulates the transfer of mannose to larger oligosaccharide chains resistant to β-elimination and the transfer of a second mannosyl unit to form an *O*-glycosidically linked mannobiosyl side chain. Incubation of particulate preparations with polyprenol-mannose-[^{14}C] in the presence of Mg^{2+} and Mn^{2+} also results in the transfer of a single mannose to the protein. When non-radioactive GDP-mannose is added to this reaction mixture, however, β-elimination yields mannobiose. The mannobiose is labeled in the reducing sugar only. These results indicate that the first mannose of this mannobiosyl side chain is transferred via a lipid intermediate, but the second mannose is transferred directly from GDP-mannose. In the presence of Mg^{2+} and Mn^{2+}, mannose apparently is also transferred from polyprenol-mannose-[^{14}C] to side chains which are resistant to hydrolysis.

INTRODUCTION

The role of polyprenol-linked sugars in the biosynthesis of complex carbohydrates of bacteria is well established [1]. Evidence has accumulated that similar lipids have a role in the biosynthesis of glycoproteins in eukaryotic cells [2]. In order to elucidate further the biosynthesis of the cell envelope of *Neurospora crassa*, we have investigated the role of polyprenol-linked sugars in this organism. In a previous publication [3] we reported that a cell-free membrane fraction of *N. crassa* mycelia in the presence of Mg^{2+} catalyses the transfer of mannose from GDP-mannose to a polyprenol lipid and an endogenous particulate protein acceptor(s). The mannosyl lipid was shown to be an obligatory intermediate in the transfer of the single mannosyl unit from GDP-mannose to form an *O*-glycosidic bond with the peptide chain.

In this study we report that chain lengthening of the carbohydrate portion of the glycoprotein does not require a lipid intermediate. A second mannosyl unit is transferred to the simple mannosyl protein core directly from GDP-mannose. Mn^{2+} is required for this second reaction. In addition, the presence of Mn^{2+} in reaction mixtures containing GDP-mannose leads to the transfer of mannosyl units to oligosaccharide side chains not released by β-elimination. A similar, but not identical, pathway for the biosynthesis of oligosaccharides labile in weak base has been reported in yeast [4].

RESULTS

Incubation of *N. crassa* particulate preparations with GDP-mannose-[^{14}C] in the presence of Mg^{2+} and Mn^{2+} resulted in the incorporation of radioactivity into two endogenous acceptors, a mannosyl lipid and mannosyl protein(s). As described previously [3], when Mg^{2+} is the only metal present, a mannosyl phosphoryl polyisoprenol is formed. If Mn^{2+} alone or Mn^{2+} plus Mg^{2+} are present in the reaction mixtures, a mannosyl lipid is also formed. TLC and subsequent autoradiography of the lipid-mannose-[^{14}C] formed when either Mg^{2+}, Mn^{2+}, or Mg^{2+} plus Mn^{2+} were present indicated that in each case only one mannosyl lipid was produced and that each of the lipid-mannoses-[^{14}C] produced had identical R_fs in solvent C. Mild acid hydrolysis of the mannosyl lipid formed with Mg^{2+} plus Mn^{2+} in the reaction mixture and subsequent TLC of the water-soluble product in solvent A yielded only mannose, as previously found when only Mg^{2+} was present [3]. Finally, unlike the results found with animal systems [5–7], a second extraction of the $CHCl_3$–MeOH insoluble radioactive material resulting from the reaction mixtures containing GDP-mannose-[^{14}C] and Mg^{2+} plus Mn^{2+} with $CHCl_3$–MeOH–H_2O (1:1:0.3) did not solubilize any additional radioactive material with the property of a lipid-linked oligosaccharide Collectively these results indicate that the only lipid-mannose-[^{14}C] produced when *N. crassa* particulate preparations are incubated with GDP-mannose-[^{14}C] is a mannosyl phosphosyl polyisoprenol, regardless of the divalent metal ion present. Although the lipids produced under the various conditions appear identical, the addition of Mn^{2+} to the reaction mixtures led to significantly different patterns of mannosylation of the protein(s).

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Table 1. Effect of metal ion on the β-elimination pattern of *Neurospora* glycoproteins*

Substrate	Metal ion	Radioactivity (cpm) incorporated into methanol-insoluble fraction	% Radioactivity in insoluble fraction dialysable after β-elimination
5 μM GDP-Man	Mg^{2+}	250 × 10^3	80
5 μM GDP-Man	Mg^{2+}, Mn^{2+}	206 × 10^3	35
Lipid-mannose-[^{14}C]	Mg^{2+}	60 × 10^3	90
Lipid-mannose-[^{14}C] plus unlabeled GDP-Man	Mg^{2+}, Mn^{2+}	57 × 10^3	67

* Mannosyl proteins produced under standard conditions were treated with weak base and dialysed as described in Experimental. Aliquots of the concentrated dialysate as well as of the non-dialysable material were counted.

Characterization of glycoproteins

After hydrolysis of the labeled glycoproteins produced with GDP-mannose-[^{14}C] and Mn^{2+} in the reaction mixture and PC in solvents A and B, all of the radioactivity co-chromatographed with mannose. Table 1 shows the pattern of β-elimination of the mannosyl proteins produced under different conditions of reaction with either GDP-mannose-[^{14}C] or lipid-mannose-[^{14}C] as the mannosyl donors. In each case the methanol-insoluble products were treated with base and dialysed as described in Experimental. As described previously [3], with 5 μM GDP-mannose and Mg^{2+} in the reaction mixture, 80% of the incorporated mannose can be released by β-elimination, a procedure which breaks *O*-glycosidic bonds to serine and threonine [8]. In contrast, when Mg^{2+} and Mn^{2+} are present in the reaction mixture only 35% of the radioactivity is released by β-elimination. Under these conditions other enzymes apparently catalyse the transfer of mannose to oligosaccharide side chains not involving linkages to serine or threonine.

The β-elimination pattern of the mannosyl protein product is also dependent on the presence of Mn^{2+} in the reaction mixture when lipid-mannose-[^{14}C] is the mannosyl donor (Table 1). As described previously [3] with lipid-mannose-[^{14}C] and Mg^{2+} in the reaction mixture, 90% of the incorporated mannose can be released by β-elimination, while with the addition of Mn^{2+} and unlabeled GDP-mannose, only 67% of the radioactivity in the mannosyl protein is released. This suggests that as in animal and plant systems [2], the lipid-mannose-[^{14}C] is a precursor of mannosyl units linked to the protein via bonds resistant to weak bases.

The material which remains non-dialysable after β-elimination was also subjected to proteolysis and gel filtration as described in Experimental. As previously described [3] with protein products produced from either mannosyl-[^{14}C] donor in the presence of Mg^{2+}, more than 90% of the radioactivity is released as low MW glycopeptides by protease digestion. When material resistant to weak base produced in the reaction mixture containing lipid-mannose-[^{14}C], unlabeled GDP-mannose, Mg^{2+} and Mn^{2+} is subjected to protease digestion, all of the radioactivity is released as a low MW peptide(s). In contrast, when weak base resistant material produced in the reaction mixture containing GDP-mannose-[^{14}C], Mg^{2+} and Mn^{3+} is subject to proteolysis and gel filtration, *ca* 75% of the radioactivity remains in the high MW, presumably oligosaccharide, fraction. No additional radioactivity is released after a second protease treatment. This result suggests that in *N. crassa* GDP-mannose is a precursor of large oligosaccharide side chains not linked via *O*-glycosidic bonds to protein. In contrast, mannosyl lipid does not appear to be a precursor of that material.

Distribution of radioactivity released by β-elimination of mannosyl protein products, as shown by PC.

β-elimination of the mannosyl protein produced in reaction mixtures containing GDP-mannose and Mg^{2+} releases only a single mannose unit. In a previous publication [3] it was shown that a mannosyl lipid is an obligatory intermediate in the transfer of this mannose from GDP-mannose to the protein. Mannose and mannobiose are released by β-elimination when Mn^{2+} is also included in the reaction mixture. This suggests that the inclusion of Mn^{2+} leads to the elongation of the oligosaccharide moiety.

The distribution of radioactivity released by β-elimination of the mannosyl-[^{14}C] protein when lipid-mannose-[^{14}C] is the mannosyl donor was studied. When Mg^{2+} is the only metal present in the reaction mixture, only mannose is released by β-elimination of the resultant mannosyl protein. The same result is obtained when either Mn^{2+} or Mg^{2+} plus Mn^{2+} are present in the reaction mixture. However, if lipid-mannose-[^{14}C] is incubated in the presence of Mg^{2+}, Mn^{2+} and unlabeled GDP-mannose, then the resultant mannosyl-[^{14}C] protein yields both mannose and mannobiose as β-elimination products. Since the possible formation of a mannobiosyl lipid was ruled out by experiments described above, the second mannose is transferred in a sequential manner either via a second mannosyl lipid or directly from GDP-mannose. The evidence that mannobiose-[^{14}C] was released from β-elimination of the mannosyl protein produced in the reaction mixture containing lipid-mannose-[^{14}C], Mg^{2+}, Mn^{2+} and unlabeled GDP-mannose, but was not released if the unlabeled GDP-mannose was absent, suggests that the second mannosyl residue is transferred directly from GDP-mannose. The following experiment was designed to prove this.

Distribution of radioactivity in mannobiose released by β-elimination of the mannosyl proteins

In order to distinguish further between the two possibilities for the addition of the second mannosyl unit, the mannosyl protein produced in reaction mixtures containing either GDP-mannose-[^{14}C], Mg^{2+} and Mn^{2+} or lipid-mannose-[^{14}C], unlabeled GDP-mannose, Mg^{2+} and Mn^{2+} was treated with NaOH in the presence of $NaBH_4$. After dialysis, desalting and chromatography as described above, the mannobiotol was eluted and hydrolysed. The hydrolysis products were then re-chromatographed in solvent A. Table 2 shows that while both the reducing sugar (mannitol) and the terminal

Table 2. Distribution of radioactivity in mannobiotol released by β-elimination from mannosyl proteins*

Substrate	Hydrolytic products after reductive β-elimination	Radioactivity (cpm)	Percentage
GDP-mannose-[^{14}C]	mannose	4160	85
	mannitol	735	15
Lipid-mannose-[^{14}C]	mannose	—	0
	mannitol	3100	100

* Mannobiotol obtained by chromatography of the dialysable products of reductive β-elimination of mannosyl proteins was hydrolysed as described in Experimental, chromatographed in solvent A. The mannose and mannitol peaks were then eluted and counted.

sugar (mannose) were labeled when GDP-[^{14}C]-mannose was the mannosyl-[^{14}C] donor, only the reducing sugar (mannitol) was labeled when the lipid-mannose-[^{14}C] was the mannosyl-[^{14}C] donor in the presence of unlabeled GDP-mannose. These results are consistent only with a scheme where the mannosyl lipid is an intermediate in the transfer of the first mannose, but where the second mannose is transferred to the mannosyl protein core directly from GDP-mannose.

DISCUSSION

As previously described [3], *N. crassa* membrane particulate preparations catalyse the transfer of mannose from GDP-mannose to a mannosyl phosphoryl polyisoprenol and mannosyl protein acceptor(s) in the presence of Mg^{2+}. In this study we have investigated the role of Mn^{2+} in the biosynthesis of *N. crassa* glycoprotein(s). While the addition of Mn^{2+} to reaction mixtures has no observable effect on the synthesis of the mannosyl lipid, it elicits several changes in the synthesis of the mannosyl protein(s). The presence of Mn^{2+} leads to the additional transfer of mannose from GDP-mannose to oligosaccharide side chains which are not subject to β-elimination; in contrast, when Mn^{2+} is absent, side chains labile to weak base are synthesized almost exclusively. Some of these resistant side chains also appear to be attached to polymers having a carbohydrate/protein ratio similar to that of yeast mannan because proteolysis released only 25% of this radioactive material as low MW glycopeptides.

The addition of Mn^{2+} to the reaction mixtures containing GDP-mannose also led to the transfer of a second mannose unit to the simple *O*-glycosidic linked mannosyl protein core. While the first mannosyl unit of this disaccharide side chain is transferred via a mannosyl lipid [3], the second mannosyl unit was shown to be transferred to the glycoprotein directly from GDP-mannose. These results, in conjunction with our previous findings [3], indicate that the following reactions occur in *N. crassa* membrane preparations:

(1) GDP-Man + polyisoprenol-P $\xrightarrow{Mg^{2+} \text{ or } Mn^{2+}}$ mannosyl-1-phosphoryl polyisoprenol + GDP;

(2) mannosyl-1-phosphoryl polyisoprenol + OH-protein $\xrightarrow{Mg^{2+} \text{ or } Mn^{2+}}$ Man-*O*-protein + polyisoprenol-P;

(3) GDP-Man + Man-*O*-protein $\xrightarrow{Mn^{2+}}$ Man-Man-*O*-protein + GDP.

No further mannosyl units appear to be added to these mannobiosyl side chains. This is in contrast to the yeast system [4] where mannotetraose side chains are apparently synthesized. The existence of mannosyl transferases which have specific divalent metal ion requirements has been observed in both yeast [4] and mammalian [9] systems.

Neurospora and yeast [4] are the only organisms in which this particular sequential synthesis of *O*-glycosidic linked oligosaccharide side chains has been elucidated. The enzymes which transfer mannose from GDP-mannose and possibly from mannosyl lipid to weak base resistant side chains on *N. crassa* glycoprotein(s) have not been studied, although they may be analogous to those studied in animal and plant systems [2].

EXPERIMENTAL

Materials. N. crassa wild type strain RL3-8A was grown from a conidial inoculation for 16 hr at 30° on a rotary shaker in 2 l. conical flasks containing 1 l. of Vogels medium N [10] using 2% sucrose as the carbon source. Mycelia were stored at −20° for <1 week before use. GDP-mannose-[U-^{14}C] (221 mCi/mmol) was obtained from the New England Nuclear Corp. Ammonyx, a nonionic detergent, was obtained from the Onyx Chemical Co. Unlabeled GDP-mannose and the proteases were obtained from Sigma. Radioactivity and protein measurements as well as the particulate enzyme preparation were performed as described previously [3].

Incorporation of mannose-[^{14}C] into lipid and protein. Incorporation of mannose-[^{14}C] was carried out in reaction mixtures containing 0.1 M Tris–Cl, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$ and 5 μM GDP-mannose-[^{14}C] (50 μCi/μmol), unless stated otherwise. When the mannosyl lipid was the only product required, no $MnCl_2$ was added. Reactions were started by the addition of particulate prepn and were carried out at 22° for 1 hr for mannosyl protein synthesis and for 20 min for mannosyl lipid synthesis. Reactions were stopped at the indicated time by the addition of 20 vol. $CHCl_3$–MeOH (2:1) with mixing. The mixture was allowed to stand for 15 min and was then centrifuged. The supernatant containing the lipid was removed and washed first with 1 vol. and then twice with 0.75 vol. 0.9% NaCl–MeOH(2:1). The washed lipid phase was then dried under N_2 and stored at −20°. The pellet from the original $CHCl_3$–MeOH extraction containing the mannosyl protein was washed once with 1 ml MeOH, twice with 1 ml 80% MeOH satd with KCl, once again with 80% MeOH and finally with H_2O.

Transfer of mannose-[^{14}C] from mannosyl lipid to mannosyl protein. Assay mixtures contained 3×10^5 cpm of partially purified mannosyl lipid transferred in $CHCl_3$–MeOH, dried under N_2 and dispersed by sonication in 25 μl of 0.4% Ammonyx. The final reaction mixture contained 0.1 M Tris–Cl, pH 7.4, 20 mM $MgCl_2$ particulate enzyme prepn (10 mg/ml) and where described 5 mM $MnCl_2$ and 200 μM GDP-mannose. Reactions were started by the addition of enzyme and were carried out at 22° for 1 hr. The reaction was stopped by the addition of 20 vol. of $CHCl_3$–MeOH(2:1), and mannosylated product(s) and substrate were separated as described above.

Hydrolytic methods. Mild acid hydrolysis of the mannosyl lipid as well as strong acid hydrolysis of the glycoprotein, mannobiose and mannobiotol were performed as described in ref. [3]. Mild base treatment of the mannose-[^{14}C]-containing protein(s) was also performed as described in ref. [3].

Alkaline borohydride treatment of the mannose-[^{14}C]-containing protein(s). The mannosyl-[^{14}C] proteins were suspended

in 1.5 ml of 0.5 M $NaBH_4$, 0.1 M NaOH for 24 hr at 25°. The reaction was stopped by the addition of 17 M HOAc and the reaction mixture was evapd under red. pres. The residue was resuspended in 1 ml of H_2O, dialysed, neutralized with Dowex 50-H^+ as previously described [3] and chromatographed in solvent A. The radioactive peak corresponding to mannobiotol was then eluted from the paper and hydrolysed with 3 N HCl. The hydrolysate was neutralized on a small column of Dowex 1-X8 formate and chromatographed in solvent A. The radioactive peaks corresponding to mannose and mannitol were then eluted and counted.

Protease digestion of the non-dialysable mannosyl protein after β-elimination. *Ca* 25 × 10^3 cpm mannose-[^{14}C]-containing protein prepared from reaction mixtures containing Mn^{2+}, Mg^{2+} and GDP-mannose-[^{14}C] or lipid-mannose-[^{14}C] was treated with proteases as previously described [3]. Gel filtration of the proteolytic digest was as described in ref. [3] except that a Sephadex G-100 column (1.6 × 58 cm) was used.

Descending PC solvents were (A) EtOAc–Py–H_2O(12:5:4) and (B) *iso*butyric acid–NH_4OH–H_2O(59:4:39). Mono- and disaccharide standards were detected after PC by the method of ref. [12].

TLC of the mannosyl lipid was carried out on Si gel G in (C)$CHCl_3$–MeOH–H_2O(65:25:4). The radioactive zones were detected by autoradiography using DuPont Cronex 2DC X-ray film.

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