A LIPID INTERMEDIATE IN MANNAN BIOSYNTHESIS IN YEAST

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Lipid intermediates in the synthesis of bacterial cell wall polysaccharides (1,2,3) have been identified as C_{55}-polyisoprenol to which the cell wall precursor is linked either via a pyrophosphate bond (4,5) or a phosphodiester bridge (3).

Recently Behrens and Cabib (6) have shown that a particulate enzyme preparation from Saccharomyces carlsbergensis incorporates mannose from GDP-mannose into mannan. The authors did not obtain, however, any indication for the involvement of a lipid intermediate in mannan biosynthesis in yeast.

The results reported here indicate that also in yeast (S. cerevisiae) as in M. lysodeikticus (3) a lipophilic mannosyl intermediate is the immediate precursor for mannan biosynthesis. Recently very similar observations have also been made for a polysaccharide synthesizing in vitro system of higher plants (Kauss, personal communication).

Materials and Methods

S. cerevisiae (strain 66,24, Fleishmann Lab.) was grown in the medium of Ghosh et al. (7) and harvested during the first half of the log phase. The washed cells were ground with aluminium oxide in Tris/HCl o,o5 M pH 7,7 in the presence of 1 mM mercaptoethanol. After centrifugation at looo × g the supernatant was spun at 48 ooo × g for 2o min; the pellet was

resuspended in grinding medium. GDP-mannose-^{14}C was obtained from NEN (Boston), GDP-^{14}C and GMP-^{14}C from the Radiochem.Centre (Amersham). The incubation conditions are given in the legends. The total incubation mixture was chromatographed on Whatman 1 in ethyl acetate/butanol/H_2O/acetic acid = 3:4:4:2,5. The radioactivity remaining at the origin increased linearly with time for several minutes (fig.1). It was hydrolyzed after prolonged treatment ($>$ 1 hr) with 1 N HCl yielding mannose-^{14}C as the sole radioactive product. Treatment with 1 N HCl for 3o min solubilized 85 % of the radioactivity; 34 % of it having a MW larger than 5ooo (Sephadex G 25). By this treatment a di- and trisaccharide was obtained (separation on Sephadex G 15), which after reduction with $NaBH_4$ yielded mannose-^{14}C and mannitol-^{14}C. - The radioactivity was determined with a Scintillation counter (efficiency about 7o %) except for the experiment of table 1.

Results

1) Formation of a mannosyl-lipid and its turnover.

It was noticed that the particulate fraction from S. cerevisiae incorporated radioactivity from GDP-mannose-^{14}C not only into a polysaccharide but also into a lipid-like material which on paperchromatograms moved with the solvent front. If this lipophilic material were an intermediate in mannan biosynthesis it was expected to posses the following properties: a) its synthesis should precede polysaccharide formation and it should constitute only a small pool, which becomes rapidly saturated with radioactivity from GDP-mannose-^{14}C; b) the ^{14}C-mannosyl-residue of the lipid should show turnover.

In fig.1 the kinetics of mannose-^{14}C incorporation from GDP-

mannose-^{14}C into the lipid and mannan are shown. The data are in agreement with the properties expected from an intermediate. The turnover of the ^{14}C-mannosyl residue of the lipophilic substance has been demonstrated in two different ways, i.e. in an experiment using phosphodiesterase (fig.2) and by a dilution experiment with non-radioactive GDP-mannose. Fig.2 shows the increase in radioactivity of the polysaccharide from 15 min (A) to 25 min (B), the radioactivity in the lipid does not significantly change during this time. Is phosphodiesterase added, however, after 15 min the radioactivity in the lipophilic substance drastically decreases. Most of the residual GDP-mannose-^{14}C is hydrolyzed to mannose-^{14}C-1-phosphate (see C). When the reaction was terminated by boiling for 2 min and phosphodiesterase was added afterwards the radioactivity in the lipid did not show any decrease. In a parallel experiment an amount of non-radioactive GDP-mannose was added at 15 min which corresponded to a 55 fold dilution of the GDP-^{14}C-mannose. Here again the radioactivity of the lipid decreased to 175 cpm.

2) Metal ion requirement of the reaction.

Evidence for the intermediate role of the mannosyl-lipid in mannan biosynthesis came from studies of the metal ion requirement of the total reaction. In agreement with Behrens and Cabib (6) it was found that mannan biosynthesis is dependent an Mn^{++}, which is only poorly replaced by Mg^{++}. Also the incorporation of ^{14}C-mannose into the lipid requires metal ions, however, Mg^{++} seems to fully replace Mn^{++}. It was noted in these experiments that the steady state level of the ^{14}C-mannosyl-lipid was relatively low in the presence of Mn^{++} cencentrations which were optimal for mannan biosynthesis. It would be in good

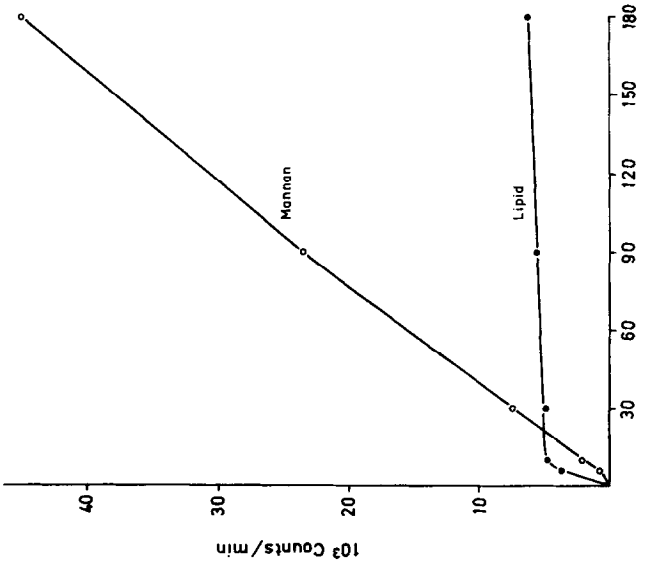

Fig.1. Time course of mannose-^{14}C incorporation into mannan and the mannosyl-lipid. Protein 0,35 mg, GDP-Man-^{14}C 0,16 µC, $MgCl_2$ 2 mM, $MnCl_2$ 1 mM; other conditions as in fig.3.

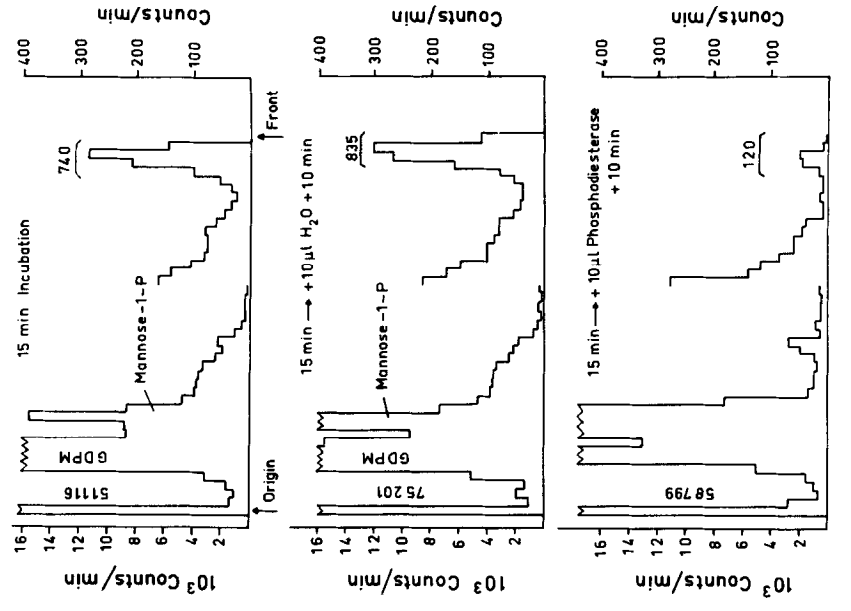

Fig.2 (right). Turnover of the mannosyl-lipid (paperchromatograms). Protein 25 µg, GDP-Man-^{14}C 1 µC, other conditions as in fig.1.

agreement with the postulated role of the lipid if it were possible to show that the addition of Mn^{++} to an incubation mixture containing Mg^{++} only would lead to an increased rate of mannan synthesis and at the same time to a decrease in the steady state level of ^{14}C-mannosyl-lipid. This is indeed the case as shown in fig.3.

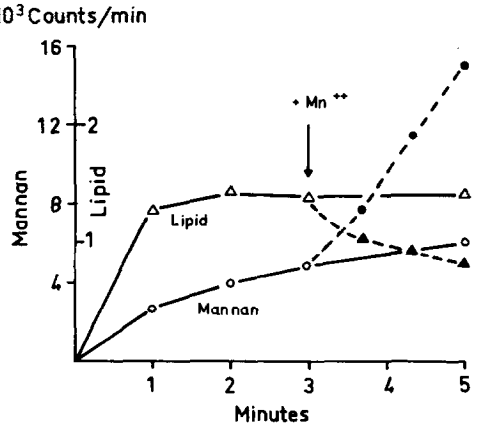

Fig.3. The effect of Mn^{++} on the rate of mannan biosynthesis and the steady state level of the mannosyl-lipid. Conditions: Tris/HCl o,o5 M pH 7,7, mercaptoethanol o,5 mM, protein 9o µg, GDP-Man-^{14}C o,1 µC (s.A. 156 mC/mmole), $MgCl_2$ 3mM; total volume 5o µl; temp.27°. Where indicated 8 mM $MnCl_2$ were added. The reaction was terminated with 5o µl acetic acid. To the control acetic acid was added at 0 time.

3) Exchange reactions.

Although the exact nature of the intermediate is not yet known some information on the linkage of the mannosyl residue was obtained by following exchange reactions. As shown in table 1 the particulate preparation catalyzed an exchange reaction between GDP-^{14}C, but not GMP-^{14}C, and GDP-mannose. In addition, when non-radioactive GDP (o,1 µmole) was added to particles previously incubated with GDP-mannose-^{14}C the radioactivity in the lipophilic substance was decreased to less than 1o % of the control; again GMP had no effect. These results clearly show

that the mannosyl residue of the lipid intermediate posesses a transfer potential comparable to that of GDP-mannose. In addition in analogy to the results of Scher et al. (3) it is suggestive that the linkage might be a phosphodiester. In agreement with these assumptions is the high acid lability of the compound; o,ol N HCl hydrolyzed the ^{14}C-mannosyl-lipid in 3o min at loo°

Table 1: GDP-Man/GDP-^{14}C Exchange Reaction.

Additions	Counts/min in GDP-Man
1) GDP-^{14}C + GDP-Man	4,188
2) GDP-^{14}C (Control)	252
3) GMP-^{14}C + GDP-Man	499
4) GMP-^{14}C (Control)	597

The reaction mixture contained in a total volume of 5o µl: Tris-HCl pH 7,7 o,o5 M, mercaptoethanol o,5 mM, Mg^{++} 2mM, Mn^{++} 1mM, 5 mµ moles of GDP-mannose (controls without), 6o µg protein, o,25 µC GDP-^{14}C (s.A.54o mC/mmole) or o,25 µC GMP-^{14}C (s.A.293 mC/mmole), respectively. The incubation was carried out at 26° for 5 min. Then 4 µg of alkaline phosphatase (Boehringer) was added to destroy residual GMP-^{14}C and GDP-^{14}C. After additional 5 min the total reaction was stopped by adding 5o µl of acetic acid. The total reaction mixture was put on Whatman Nr.3 MM and separated by electrophoreses (2o volts/cm; o,2 M ammonium formate pH 3,7). The radioactivity moving with GDP-Man was measured with a methane flow counter with window directly on paper (efficiency 7 %). The radioactive compound of sample 1 was rechromatographed in iso-butyric acid - NH_4OH (conc.) - water (57:4:39, by vol.) and in ethanol-1 M ammonium acetate pH 7,5 (5:2, by vol.); the total radioactivity coincided with GDP-Man.

almost completely. 87 % of the total radioactivity was recovered in free mannose after this treatment.

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