Yeast manno-protein biosynthesis: Solubilization and selective assay of four mannosyltransferases

(multimannosyltransferases/enzyme solubilization/mannan biosynthesis)

TASUKU NAKAJIMA AND CLINTON E. BALLOU*

Department of Biochemistry, University of California, Berkeley, Calif. 94720

Contributed by Clinton E. Ballou, July 24, 1975

ABSTRACT

Using appropriate yeast strains and exogenous acceptors, we have devised specific assays for four mannosyltransferase activities involved in biosynthesis of the carbohydrate outer chain of yeast manno-proteins. The assays utilize GDP-[14C]mannose as the donor and unlabeled oligosaccharides as the acceptors, the products being neutral radioactive oligosaccharides one mannos unit larger than the radioactive acceptors. The multiglycosyltransferase system from Saccharomyces cerevisiae was solubilized in Triton X-100 and urea and purified 100-fold. Free mannose is an acceptor for the α1→2-mannosyltransferase, the major product being α1→2-[14C]Man→3-Man. The α1→6-mannooligosaccharides serve as acceptors for both the α1→2- and α1→6-transferases, but the tetrasaccharide αMan→2-Man→2αMan→3-Man is a specific acceptor for the latter enzyme and yields αMan→2αMan→3-Man→2Mannitol. The multienzyme system works in concert to make "mannan" in a cell-free in vitro system.

Yeast cell wall manno-proteins are a heterogeneous family of complex glycoproteins. The carbohydrate, mostly mannoose, is attached to the protein in two ways—as short oligosaccharides linked to serine and threonine, and as highly branched polysaccharide chains attached to asparagine through a di-N-acetylglucosamiose unit (1). By genetic and chemical analysis, it was shown recently that the polymer chains consist of an inner core of 12-15 mannose units and

MATERIAL AND METHODS

Materials. Saccharomyces cerevisiae X2180 mnn1, mnn2, and mnn4 mutants were provided by D. L. Ballou (3) and Saccharomyces chevalieri was a gift from Dr. H. J. Phaff. GDP-[14C]mannose (160 Ci/mol) and sodium bovotride (180 Ci/mol) were from New England Nuclear, and unlabeled GDP-mannose came from Sigma. Bio-Gel P-2 (-400 mesh), P-6 (200-400 mesh), Dowex AG1-X8, and Cell Lex (DEAE-cellulose) were obtained from Bio-Rad.

α1→6-Mannooligosaccharides were prepared by partial acetylation of α1→6-mannan backbone followed by gel filtration (2). α1→2-Mannooligosaccharides, and mannottetrose, αM→3αM→4αM→5M, were prepared by acetylation of S. chevalieri (4) and S. cerevisiae mannans (5), respectively. α1→3-Mannobiose was a gift from Dr. L. Rosenfeld. Inner core fragment from S. cerevisiae X2180-1A5 mnn2 mutant was prepared as reported (2).

General Methods. Carbohydrate was determined with a phenol-sulfuric acid reagent and protein by a modified Lowry method (6). Acid hydrolysis, acetylation, acetylation, and reduction of mannan or mannoooligosaccharides were done as described elsewhere (2).

Descending paper chromatography was done on Whatman no. 1 paper in the following solvents: A, ethyl acetate–pyridine–water (5:3:2); B, ethyl acetate–pyridine–water (8: 2:1); C, ethyl acetate–actic acid–formic acid–water (18:3: 1:4). Paper electrophoresis was done on Whatman no. 1 paper in 50 mM sodium borate pH 9.2 at 20 V/cm for 8 hr. Sugars and sugar alcohols were detected with alkaline silver nitrate. Radioactivity was measured on a Packard Radiochromatogram Scanner or a Packard Tri-Carb liquid scintillation counter.

Preparation of Partially Purified Mannosyltransferases. S. cerevisiae cultures were grown for 12 hr at 30° with shaking in 3 liters of medium containing 2% D-glucose, 1% yeast extract, and 2% peptone in three 2-liter Fernbach flasks. All steps described below were carried out below 4° unless stated otherwise. The cells were harvested by centrifugation, washed twice with cold 1% KCl and once with 0.1 M Tris-HCl pH 7.2 containing 0.01 M dithiothreitol. The washed cells (5 g) were suspended in 10 ml of 0.1 M Tris-HCl pH 7.2, containing 1 mM dithiothreitol and then broken by grinding with 18 g of aluminum oxide in an ice-cold mortar for 10 min. The homogenate was fractionated by centrifugation at 4000 × g for 10 min to remove cell debris, and then at 100,000 × g for 60 min. The latter particulate fraction was treated 1 hr with 5 ml of buffer consisting of 1.5% Triton X-100 and 2 M urea (7) in 0.1 M Tris-HCl pH 7.2. The solubilized mannosyltransferases were obtained in the supernatant by centrifugation at 100,000 × g for 60 min.

The 100,000 × g supernatant fraction, 38 mg of protein in the solubilizing buffer, was diluted 5-fold with distilled

Abbreviations: M, D-mannose; rM, mannotetitol; M2, mannoobiase; rM2, reduced mannoobiase; αM→3M, α1→2-linked mannoobiase; GlcNaC, N-acetyl-D-glucosamine; mnn, the designator for mutations in genes concerned with mannan biosynthesis

* To whom correspondence should be addressed.

3912
Table 1. Purification of the mannosyltransferase system

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,000 x g supernatant of homogenate</td>
<td>1840</td>
<td>0.45</td>
<td>100</td>
</tr>
<tr>
<td>100,000 x g supernatant of homogenate</td>
<td>1750</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Triton X-100/urea extract of 100,000 x g pellet</td>
<td>38</td>
<td>769</td>
<td>19.4</td>
</tr>
<tr>
<td>Triton X-100/urea-insoluble pellet</td>
<td>6.3</td>
<td>41</td>
<td>6.6</td>
</tr>
<tr>
<td>DEAE-cellulose column fractions 80–105</td>
<td>3.1</td>
<td>151</td>
<td>48.7</td>
</tr>
</tbody>
</table>

One unit of enzyme is the amount that will incorporate 1 pmol of mannose from GDP-[14C]mannose into exogenous acceptor per min/mg of enzyme protein when assayed under the standard conditions.

water and applied to a DEAE-cellulose column (2 x 10 cm) equilibrated with 0.02 M Tris-HCl pH 7.2 containing 1.5% Triton X-100 and 5% glycerol. The mannosyltransferase activities were eluted with a linear gradient formed with 300 ml of 0.02 M Tris-HCl pH 7.2 containing 1.5% Triton X-100 and 5% glycerol in the mixing chamber and 300 ml of 0.6 M Tris-HCl pH 7.2 containing 1.5% Triton X-100 and 5% glycerol in the reservoir. Fractions of 5 ml were collected and assayed. Active fractions 80–105 were concentrated to 3 ml in an Amicon ultrafilter with a PM-10 membrane, and the concentrate was dialyzed for 6 hr against 500 ml of 0.1 M Tris-HCl containing 5% glycerol.

Standard Assay of Mannosyltransferase Activities. Incubation mixtures contained 50 mM Tris-HCl buffer pH 7.2, solubilized enzyme from S. cerevisiae mann4 mutant (10–30 µg of protein), 10 mM MnCl2, 0.6 mM GDP-[U-14C]mannose (4000–6000 cpm), and 2 mM exogenous acceptor in a final volume of 50 µl. For assay of α1→2-mannosyltransferase activity, mannose was used as acceptor; for the α1→3-mannosyltransferase-I we used reduced α1→2-mannotriose, αM→αM→αM; and for α1→6-mannosyltransferase S. cerevisiae mannnotetraose, αM→αM→αM→αM, was used. The additional α1→3-mannosyltransferase-II activity from S. chevalieri was assayed using reduced mannnotetraose, αM→αM→αM→αM. The reaction mixtures were incubated for 30 min at 25°, then terminated; excess GDP-[U-14C]mannose was removed by passing the solution through a Dowex 1-X8 column (0.5 x 5 cm). The neutral products were eluted with 1 ml of water and the radioactivity was counted in 10 ml of Bray’s solution.

Acceptor Km values were determined in presence of 0.6 mM GDP-mannose, and donor Km values in presence of 2 mM acceptor. The constants were estimated from Lineeweaver-Burk plots of the data. The pH-dependence of the transferase reactions was determined on enzyme that was dialyzed against 0.01 M Tris-HCl buffer at pH 7.0 for 4 hr. The dialyzed solution was diluted prior to assay with an equal volume of 0.4 M buffer of the appropriate composition. Divalent cation requirements were determined on enzyme that had been dialyzed for 3 hr against 0.01 M EDTA in 0.1 M Tris-HCl buffer at pH 7.0 and for 12 hr against the same buffer without EDTA.

Structural Characterization of Mannosyltransferase Products. For product analysis, reactions were scaled up 5-fold over the standard assay and a 2-hr incubation was used. The labeled product was purified on paper in Solvent A or on a Bio-Gel P-2 column (1 x 100 cm). The recovered fractions were analyzed by partial acid hydrolysis and by acetylation. For acetylation, the reaction was done at 40° for 12 hr (9) with unlabeled wild-type mannan added as a carrier, and the recovered deacetylated products were separated on a Bio-Gel P-2 column. For partial acid hydrolysis, the samples were treated in 0.3 M HCl at 100° for 3 hr. The hydrolysates were fractionated on a Bio-Gel P-2 column (1 x 50 cm), and the radioactive disaccharide components were characterized by chromatography in Solvent C or by electrophoresis in borate buffer. Radioactive disaccharides were hydrolyzed with a bacterial exo-α-mannanase (10), and the products were chromatographed in Solvent B.

Synthesis of Mannan Polymer with Exogenous Acceptor. The incubation mixture contained solubilized mannosyltransferase (3 mg of protein), 100 mM unlabeled GDP-mannose, 1 mM α1→6-[3H]mannopentaotol prepared by reduction of the mannopentaose with NaBGT4 and 100 mM MnCl2 in 100 µl of 0.1 M Tris-HCl buffer pH 7.5. The reaction was followed by passing samples through a Dowex 1-X8 column (0.5 x 5 cm), after which the effluents were fractionated on a Bio-Gel P-6 column (1 x 50 cm). The labeled products recovered from the Bio-Gel P-6 were acetylated, and the deacetylated fragments were reduced with NaBGT4 and then chromatographed on paper in Solvent A for 17 hr.

RESULTS

Preparation of the solubilized mannosyltransferase system

The extraction system of Garewal and Wasserman (7) provided a stable enzyme preparation amenable to the usual protein purification procedures. Table 1 summarizes steps that led to a 100-fold purification. Such preparations retained at least 50% of their activity for days when stored at 0° in the presence of 5% glycerol.

All three mannosyltransferase activities were eluted together from the DEAE-cellulose column, and when assayed individually the reactions were linear for at least 60 min. The α1→6-transferase activity was stable during incubation at 30° for at least 2 hr, but inactivation occurred at higher temperature. All three transferases had broad optima at pH 7–8 in Tris-HCl buffers, whereas phosphate buffers inhibited the reaction. The reactions were activated about 50% by 10 mM Mn2+ before or after dialysis against EDTA. Mg2+ had no effect.

Demonstration of the multiplicity of mannosyltransferase activities

Fig. 1 illustrates the formation of several products, the structures being dependent on the acceptor but in each instance being one mannone unit larger. Under the assay conditions, the product is at too low a concentration to act as an acceptor for further additions. That different linkages are formed in each reaction is suggested by the results in Fig. 2. The gel filtration properties of the products and of their partial acetylates show that some have acetylation-stable linkages whereas others are degraded by this reaction.
Specificity of the mannosyltransferase assays

By selection of mutants or acceptors, we have limited the specific transferase activity that is expressed in any one assay. Thus, mannose acts as an acceptor for the α1→2-mannosyltransferase as demonstrated by the formation of α1→2-[14C]mannobiase (Table 2). In contrast, α1→6-mannooligosaccharides are acceptors both for the α1→2- and α1→6-mannosyltransferases, as shown by the formation of both [14C]mannose and [14C]mannobiase on partial acetolysis of the product (Fig. 2B). It is probable that the [14C]mannobiase is formed by transferases involved in synthesis of both the outer chain and the inner core because some mannobiase-yielding product was formed with this acceptor when using enzyme prepared from the mna2 mutant. This mutant has a defective α1→2-transferase-I and makes mannan with an unbranched outer chain (11).

A specific assay for the α1→6-enzyme in S. cerevisiae uses the mannotetraose αM→βαM→βαM→βM, a “completed” mannan sidechain that can only accept a mannose unit in 1→6 linkage (Fig. 2A and Table 2). S. cerevisiae mannan, however, contains a pentasaccharide sidechain with a second α1→3-linked mannose unit (4), and enzyme prepared from this yeast utilizes the reduced mannotetraose to form a mannopentaose product (Fig. 2D). Finally, the α1→3-mannosyltransferase-I of S. cerevisiae is assayed with

FIG. 2. Gel filtration on Bio-Gel P-2 of the radioactive products from the mannosyltransferase system with GDP-[14C]mannose as the donor and various acceptors indicated on the figure. Radioactive products of the enzymic reaction are indicated with a solid line, and the radioactive products formed by partial acetolysis of the enzymic products are indicated with a dashed line. Reduced oligosaccharides have elution volumes one sugar unit larger than the unreduced parent compound.

FIG. 3 (left). Fractionation on a Bio-Gel P-6 column of the products formed at different times by incubation of the solubilized mannosyltransferase system with GDP-mannose and α1→6-[P]mannopentaitol acceptor. The elution positions of the acceptor, a reference oligosaccharide of about 15 mannose units, and the void volume, Vo, are indicated. The sharpening of the peak of the 120-min product suggests that the material exceeds the exclusion limit of the gel.

FIG. 4 (right). Paper chromatography of partial acetolyses of the “mannan” synthesized in Fig. 3. The products isolated by gel filtration were subjected to partial acetolysis, after which the deacetylated and NaBT4-reduced fragments were chromatographed on paper. The figure shows the radioactive scans for incubations of 30 min (top), 60 min (middle), and 120 min (bottom). Reference strips show the positions of migration from right to left of mannotol, mannobiose, mannotriose, and mannotetraose.
Table 2. Characterization of the products from mannosyltransferase reactions

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Enzymic product</th>
<th>Acetolysis product</th>
<th>Acid hydrolysis products</th>
<th>Radioactive product from α-mannanase digestion</th>
<th>Structure of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>M₁</td>
<td>M₂</td>
<td>M → M₂M</td>
<td>αM₄M⁻⁺αM⁻⁺M</td>
<td>M₄</td>
</tr>
<tr>
<td>αM⁻⁺αM⁻⁺M</td>
<td>rM₄</td>
<td>rM₄</td>
<td>rM₄ → rM₄M</td>
<td>αM₄M⁻⁺αM⁻⁺M</td>
<td>M₄</td>
</tr>
<tr>
<td>αM⁻⁺αM⁻⁺αM⁻⁺M</td>
<td>M₅</td>
<td>M₅ + M₇</td>
<td>M → M₂M</td>
<td>αM₄M⁻⁺αM⁻⁺M</td>
<td>M₄</td>
</tr>
<tr>
<td>αM⁻⁺αM⁻⁺αM⁻⁺αM⁻⁺M</td>
<td>M₆</td>
<td>M + M₇</td>
<td>M → M₂M</td>
<td>αM₄M⁻⁺αM⁻⁺M (40%)</td>
<td>M₄</td>
</tr>
<tr>
<td>αM⁻⁺αM⁻⁺αM⁻⁺αM⁻⁺αM⁻⁺M</td>
<td>M₇</td>
<td>M + M₇</td>
<td>M → M₂M</td>
<td>αM₄M⁻⁺αM⁻⁺M (60%)</td>
<td>M₄</td>
</tr>
<tr>
<td>αM⁻⁺αM⁻⁺αM⁻⁺αM⁻⁺αM⁻⁺αM⁻⁺M</td>
<td>M₈</td>
<td>M + M₇</td>
<td>M → M₂M</td>
<td>αM₄M⁻⁺αM⁻⁺M</td>
<td>M₄</td>
</tr>
</tbody>
</table>

The [¹⁴C]mannose incorporated in the enzymic reaction is identified as M*.

reduced α₁→2-mannotriose, αM⁻⁺αM⁻⁺αM⁻⁺M, the product being a reduced tetrasaccharide (Fig. 2C). Because such a product was not formed by the mnn₁ mutant, which lacks terminal α₁→3-linked mannose units in its mannan (11), the tetrasaccharide must possess the α₁→3 linkage.

Preliminary evidence for the linkages formed by the different mannosyltransferases came from partial acetolysis studies. Thus, the products from the α₁→2- and α₁→3-mannosyltransferase reactions were stable to this treatment, whereas the α₁→6-transferase product was degraded to free [¹⁴C]mannose. Direct evidence for the assigned linkages came from the structures of the radioactive disaccharides formed by partial acid hydrolysis (Table 2). In each instance, the labeled disaccharide had the linkage consistent with the specificity of the transferase being assayed.

Cell-free biosynthesis of "mannan"

Purified enzyme from S. cerevisiae mnn₄ mutant, incubated with a high concentration of unlabeled GDP-mannose and α₁→6-[³H]mannopentaitol, gave a radioactive product (Fig. 3) of progressively increasing size with time of incubation, reaching at least 35 mannose units after 2 hr. The acetolysis pattern (Fig. 4) shows only mannose at 30 min, a consequence of the action of the α₁→6-transferase, whereas mannobiose is observed after 1 hr owing to action of the α₁→2-transferase. The absence of larger acetolysis-stable fragments indicates that the second α₁→2-transferase (11) is not very active in this preparation; consequently, the α₁→6-transferase lacks a suitable acceptor.

Preliminary kinetic parameters of the mannosyltransferase system

The relative acceptor activities of a number of manno oligosaccharides are given in Table 3. The α₁→6-linked homologs, which can serve as acceptors for both the α₁→2- and α₁→6-transferases, show good activity up to the heptasaccharide, at which the activity drops sharply. The reduced α₁→2-linked series shows an unusual pattern, the disaccharide having low activity, the trisaccharide a high activity, and the tetrasaccharide being inactive. We have shown that the reduced trisaccharide is an acceptor for the α₁→3-transferase-I. We suspect that the reduced disaccharide may be a poor acceptor for an α₁→2-transferase. The inactivity of the reduced α₁→2-tetrasaccharide correlates with the fact that S. cerevisiae mannan has no sidechain with three consecutive 1→2 linkages.

DISCUSSION

Yeast mannan biosynthesis has been studied by Behrens and Cabib (12), who described the incorporation of labeled mannose from GDP-mannose into endogenous acceptors to produce mannan-like material. Subsequently, Lehle and Tan-
(13) found that mannose, mannobiose, and mannotriose can act as exogenous acceptors, and that the disaccharide formed from mannose had the α1→2 linkage. Farkas et al. (14) reported similar results. Sharma et al. (15) have shown that the first mannose added to serine and threonine in manno-proteins comes from mannosyl dolichol phosphate, and that subsequent mannose units probably are derived directly from GDP-mannose.

In this study, we have designed specific assays for four of the mannosyltransferases in yeast mannan biosynthesis, using exogenous acceptors and a solubilized enzyme system. The complexity of the carbohydrate component in yeast manno-proteins (Fig. 5) suggests that a minimum of 10 mannosyltransferases must be involved in its biosynthesis. Genetic evidence indicates that formation of all of the terminal 1→3-linked mannose units is controlled by a single gene, because a mutant (designated mnn1) simultaneously lost this component in all positions (11). By direct assay, we have now demonstrated this enzymatic activity in wild-type S. cerevisiae and its absence in the mnn1 mutant.

The mannotetraose unit is the longest sidechain in S. cerevisiae mannan. However, several Saccharomyces species, intertectile with S. cerevisiae, make mannan with a pentasaccharide sidechain by adding another mannose in α1→3 linkage (4). We have now demonstrated this α1→3-mannosyltransferase-II activity in S. chevalieri extracts.

The first sidechain mannan unit in S. cerevisiae mannan is attached to the α1→6-linked backbone by an α1→2 linkage. The mnn2 mutant, which makes mannan with an unsubstituted outer chain, has now been shown to lack the α1→2-mannosyltransferase-I associated with this structure. Some acetolysis-resistant disaccharide product is made by extracts from this mutant, which probably reflects the activity of an inner core transferase. The α1→2-transferases that make the oligosaccharides attached to the hydroxymino acids are not expected to act on the α1→6-mannooligosaccharide acceptors.

The α1→6-mannosyltransferase synthesizes the mannan backbone, and a demonstration of this enzyme activity with an exogenous acceptor has not been reported previously in yeast, although a similar enzyme activity is found in Cryptococcus laurentii (16). In our system, this transferase is demonstrated by the addition of a new mannose unit in 1→6 linkage to the acceptor αM→αM→αM→αM→αM.

From preliminary kinetic studies, we have sought some hint as to how the activities of the different enzymes might be regulated to produce a mannan of characteristic size and degree of branching (Table 4). The order of affinities of the transferases for the donor GDP-mannose is α1→6 > α1→3 > α1→2, so the sugar nucleotide concentration could affect the relative activities of these three enzymes. On the other hand, the affinities for the acceptors vary considerably depending on their structures. Thus, the α1→6-transferase has a lower Km for the acceptor consisting of two sidechain fragments connected by a 1→6 linkage than it does for the single mannotetraose sidechain, a reasonable observation since the former more nearly mimics the structure of the growing end of a mannan chain. The Km of the mixed α1→2- and α1→6-transferase system for the α1→6-mannooligosaccharide acceptors decreased sharply in going from the di- to the tetrasaccharide, again probably a reflection of the closer approximation to the structure of the natural acceptor.

If the mannosyltransferases described here are involved in mannan biosynthesis, it is expected that they would act in concert to produce macromolecular material with the properties of mannan. We have shown this to occur when the enzyme preparation from S. cerevisiae wild-type is incubated for an extended time with the α1→6-mannopentaitol acceptor and a high concentration of GDP-mannose. Polysaccharide material is formed with a molecular size exceeding the exclusion limit of Bio-Gel P-6 as a result of the action of both the α1→6- and α1→2-mannosyltransferases.

This study was supported by National Science Foundation Grant GB-35299X and by U.S. Public Health Service Grant AM884.

Table 4. Km values for the different mannosyltransferases

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Transferase</th>
<th>Oligosaccharide acceptor Km (mM)</th>
<th>GDP-mannose donor Km (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αM→αM</td>
<td>α1→2</td>
<td>7.5</td>
<td>0.5</td>
</tr>
<tr>
<td>αM→αM→αM→αM</td>
<td>α1→2</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>αM→αM→αM→αM→αM→αM→αM</td>
<td>α1→2</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>αM→αM→αM</td>
<td>α1→6</td>
<td>4.5</td>
<td>0.1</td>
</tr>
<tr>
<td>αM→αM→αM</td>
<td>α1→6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>αM→αM</td>
<td>α1→3</td>
<td>2.3</td>
<td>0.18</td>
</tr>
</tbody>
</table>