Biosynthesis of Yeast Mannan

PROPERTIES OF A MANNOSYLPHOSPHATE TRANSFERASE IN SACCHAROMYCES CEREVISIAE*

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A homogenate of mechanically broken, freshly grown Saccharomyces cerevisiae X2180 cells catalyzes the transfer of mannosylphosphate units from guanosine diphosphate mannone to reduced α1 → 2-[3H]manno- tetraose to yield reduced mannosylphosphoryl [3H]-mannotetraose. The product is analogous in structure to the phosphorylated mannan side chains, which suggests that the enzyme activity is involved in mannan-protein biosynthesis in the intact cell. The mannosylphosphate transferase activity, localized in a membrane fraction obtained by differential centrifugation at 100,000 × g, was solubilized by Triton X-155 and purified 250-fold by ammonium sulfate precipitation and by ion exchange and gel filtration chromatographies. The enzyme requires Mn2+ or Co2+ ions for activity and is stimulated by various detergents.

The mnn2 and mnn3 mannan mutants of S. cerevisiae possess normal levels of mannosylphosphate transferase activity, whereas the mnn4 mutant cells contain very low, if any, activity. This is consistent with a previous conclusion that the mnn4 mutation affects the mannosylphosphate transferase activity, whereas the mnn2 and mnn3 strains possess phosphate-deficient mannans because they are unable to synthesize the appropriate side chain precursors. A new mannan mutant class with the mnn4 chemotype was isolated, but the mutation proved to be recessive and nonallelic with the mnn4 locus. This new locus is designated mnn6.

Saccharomyces cerevisiae cell wall mannanprotein contains mannosylphosphate and mannobiophosphate groups attached by a phosphodiester linkage to oligosaccharide side chains in the polysaccharide component of the glycoprotein (1-3). The function of these substituents is unknown, but they provide the major negative charge to the cell surface and appear to represent the only phosphate in the wall.

Changes observed in the mannan produced by yeast mutants with altered glycosyltransferase activities suggest that these glycosylphosphate units could be formed in a two-step process consisting of an initial transfer of the mannosylphosphate group from a sugar nucleotide to the acceptor side chain, followed by a transfer of mannone from sugar nucleotide to the mannosylphosphate group to form the α1 → 3-linked disaccharide (4). The latter step would presumably be catalyzed by the known enzyme of broad specificity that adds α1 → 3-linked mannone in several different locations in the mannan (5, 6).

Some of the S. cerevisiae mannan mutants lack phosphate in the mannanprotein because they are unable to make the appropriate acceptor side chains (the mnn2 and mnn3 classes), whereas the mnn4 mutant appears to be deficient in the mannosylphosphate transferase activity (5, 7). This latter mutation is dominant; that is, the heterozygous diploid shows the mutant phenotype. The present study was undertaken to devise an assay for the mannosylphosphate transferase activity in S. cerevisiae, to investigate the properties of the enzyme, and to elucidate the mechanism of dominance by the mnn4 mutation.

EXPERIMENTAL PROCEDURES

Materials—Lowex AG 1-2X, Dowex AG 50-X8, Cellex 1', Cellex D, Bio-Gels A-0.5m, A-1.5m, and P-2 (200 to 400 mesh) were purchased from Bio-Rad, and DEAE-Sephadex A-25 was from Pharmacia. New England Nuclear supplied sodium [3H]borohydride (300 Ci/mol) and GDP-[U-14C]mannose (210 Ci/mol). Sodium borohydride came from Alfa Inorganics, and Alcian Blue was from K and K Laboratories. The sodium salts of GDP-d-mannose, GDP-d-glucose, UDP-d-mannose, ADP-d-mannose, d-mannose 1-phosphate, d-mannose 6-phosphate, GTP, GDP, and ATP were purchased from Sigma, as well as Triton, Tween, and Brij detergents. All other chemicals were of the best grade commercially available. Solutions were prepared in glass-distilled, deionized water.

Alkaline phosphatase (EC 3.1.3.1), grade BAP (35 units/ml), was from Worthington Corp.; pronase, grade B, from Calbiochem; and Zymolyase 5000 from the Kirin Brewery, Japan. Dr Christopher Reading and Mr. Robert Cohen of this laboratory provided jack bean mannosidase (EC 3.2.1.24), specific activity 2 units/ml with a phosphatase contamination of 7 × 10^-3 units/ml at the pH optimum of the mannosidase.

General Methods—Sugars were estimated by the phenolsulfuric acid method (8), and phosphate was determined by Bartlett's procedure (9). Radioactivity was measured in a Beckman LS 3150 scintillation counter in a dioxane-based fluid (10). Corrections for quenching utilized an external channel standards ratio. Whatman No. 1 filter paper was used for descending paper chromatography in ethyl acetate:pyridine:water (5:3:2). Paper electrophoresis was done on Whatman No. 3MM paper (18 × 57 cm) in 0.05 M NaHCO3, pH 8.1, for 2 h at 1000 V on a water-cooled, flat bed apparatus. Separation, which depended on size and charge, was independent of the type of glycosidic linkage in the oligosaccharides. Sugars and sugar alcohols were detected with an AgNO3/NaOH dip reagent (11). Radioactivity was measured with a Packard radiochromatogram scanner or by cutting the paper strips into 1.0-cm segments that are moistened and counted in vials with Bray's solution (10). Protein was measured by a modification of the procedure of Lowry et al. (12), in which 10% sodium dodecyl sulfate was added to the samples before the copper reagent

* This work was supported by National Science Foundation Grant PCM74-18893 and United States Public Health Service Grant AI-12522. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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to prevent precipitation of the phenol reagent by detergent (13, 14).

Mild acid hydrolysis of glycosyl phosphodiester linkages was performed according to Thiene and Ballou (5). In alkaline phosphatase digests, 0.2-ml samples containing 0.2 unit of enzyme were incubated in 0.1 mM NH$_4$HCO$_3$, pH 8.5, for 12 hr at 37°C and α-mannosidase digestions were done for 0 hr at 37°C with 0.02 unit of enzyme at pH 5.0. For both digestions, 0.01 M NaF was added to inhibit the contaminating phosphatase. Progress of the reaction was monitored by paper chromatography or by paper electrophoresis after the protein was denatured by heat and removed by centrifugation.

Preparation of Mannan and Oligosaccharides—Mannans were extracted from cells grown to stationary phase and were purified as described by Ballou and Leloir (1). K. brevis mannan (strain X2180—dead cells) were laboratory preparations (16). To isolate phosphorylated oligosaccharides, the acetylation reaction was terminated by adding aqueous barium acetate equivalent to the sulfuric acid. After evaporation of the salts, the acetylated oligosaccharides were extracted into 70% methanol, the barium sulfate was removed, and the products were deacetylated at pH 12 in aqueous NaOH. The oligosaccharides were fractionated either on a Bio-Gel P-2 column (4 × 250 cm) by elution with water or on a Sephadex column (2 × 200 cm) by elution with a gradient of 0 to 0.5 M NH$_4$HCO$_3$.

To prepare H-labeled acceptors and reference compounds, a crystalline sample of sodium $^{[3}H]$borohydride was added to 0.14 ml of a solution of oligosaccharide (0.2 mg) in 50 mM NH$_4$HCO$_3$, pH 8.5. After 1 hr, an excess of unlabeled NaBH$_3$ was added and allowed to react for 8 h to ensure complete reduction. The reaction was terminated by adding Dowex 50-X8, the solution was evaporated, and the borate was removed by repetitive evaporation of methanol. The radioactive product was desalted on a Bio-Gel P-2 column (2 × 100 cm) and dried.

Growth of Yeast Cultures—Hansenula polymorpha 52-251 and Kluyvera brevis 55-45 were obtained from Dr. H. J. Phaff, University of Chicago, and the S. cerevisiae strains were from the laboratory collection. Saccharomyces strains were grown aerobically in liquid culture at 30°C on a medium that contained 1% yeast extract, 2% peptone, and 2% D-glucose. K. brevis and H. polymorpha cells were cultured similarly in a medium described by Stewart et al. (10). Growth was monitored by turbidity at 600 nm, and cells were harvested by centrifugation. The ability of cells to bind Alcian Blue was determined by heating it for 2 min. The solution remaining, after sedimenting the denatured protein, was applied in l-cm streaks to Whatman No. 3MM paper and electrophoresis was performed to separate the labeled components.

To prepare mannan acceptors, the al-α-mannosyltransferase [H]mannosylphosphate (20 pmol) provided by Dr. Ludwig Lehle (22, 23) was dried under nitrogen in a tube and 10 mM mannopotetraose-1, 6 μM MnCl$_2$, and 1.5 mg of particulate enzyme in 0.5% Triton X-155, and, in some tubes, 6 mM GDP-mannose, were added with mixing and sonication. The 25-μl samples were incubated for 3 h at 30°C and processed by electrophoresis as described for unlabelled oligosaccharide acceptors.

Preparation of Reaction Products for Analysis—For products containing only the tritium-labeled acceptor, eluates from small assay columns were pooled, concentrated, desalted on a Bio-Gel P-2 column (2 × 56 cm), and lyophilized. To isolate the product labeled both with $^3$H and $^14$C, the assay mixture containing solubilized enzyme was scaled up to 0.85 ml and 0.5 μM GDP-[1-14C]mannose (1.5 Ci/mol) replaced 6 μM unlabeled GDP-mannose. After a 2-h incubation, the diluted reaction mixture was heated to denature the protein, cooled, and centrifuged, and the precipitate was washed with water. The combined supernatant extracts were applied to a DEAE-Sephadex A25 (HCO$_3$) column (4 × 45 cm), which was washed with 500 ml of water and eluted with a 0.005 to 0.05 M HCl gradient (1 liter each), and the doubly labeled material was collected.

RESULTS

Mannosylphosphate Transferase Assay—The presence in S. cerevisiae of mannan of mannophosphatase units attached to α-1→6 mannooligosaccharide side chain units (24) suggested the existence of an enzyme capable of transferring the mannosylphosphate group to the mannan. An assay for this activity was designed utilizing unlabelled GDP-mannose and labeled oligosaccharide acceptors. The acidic labeled product was absorbed on a small ion exchange column, which was washed free of excess labeled acceptor before the enzymic product was eluted with acid. Potential labeled acceptors were prepared by reduction of oligosaccharides with [H]borohydride, which converted the sugar unit at the reducing end to a trinitiated polyol. The standard assay employed reduced [H]mannopotetraose-1 (Table I), αMan → βMan → βMan → βMan → βMan)nmannitol, but other acceptors were tested and are described below. Although the partially purified enzyme preparation contained other enzyme activities associated with mannoprotein biosynthesis, the α1→6 manno-oligotransferase could not act because the manno unit at the reducing end was converted to mannotriose in the reduced acceptor, and the protein solution. All fractions were dialyzed exhaustively before assay and were concentrated by vacuum dialysis.

A 5-ml sample of (NH$_4$)$_2$SO$_4$-fractionated material (30 mg of protein/ml) was fractionated on a Bio-Gel A-1.5m column (2 × 100 cm) equilibrated with 25 mM imidazole acetate, pH 6.5, and the eluted fractions were monitored for protein by carboxylic activity. Reaction mixtures routinely contained enzyme extract (0.2 to 1 mg of protein), assay buffer (25 mM imidazole acetate buffer, pH 6.5 or 7.5), 6 mM MnCl$_2$, and 6 mM GDP-mannose, 10 mM reduced [H]mannopotetraose-1 (2 to 5 Ci/mol), and 0.2 to 1% Triton X-155, in a volume of 20 to 50 μl. After incubation for 1 h at 30°C, the reaction was terminated by the addition of 1 ml of cold water and the sample was applied to a Dowex 1 (Cl-) column (0.5 × 3 cm). The column was washed with 1 ml of water and 2 ml of 0.01 N HCl, after which the labeled product was eluted with 1.5 ml of 0.05 N HCl and a 0.5-ml sample was counted for radioactivity. In control incubations, either heat-denatured enzyme was used or one of the reaction components was omitted and added after termination of the reaction.

A sample of yeast dolichyl [14C]mannosylphosphate (20 pmol) provided by Dr. Ludwig Lehle (22, 23) was dried under nitrogen in a tube and 10 mM mannopotetraose-1, 6 μM MnCl$_2$, and 1.5 mg of particulate enzyme in 0.5% Triton X-155, and, in some tubes, 6 mM GDP-mannose, were added with mixing and sonication. The 25-μl samples were incubated for 3 h at 30°C and processed by electrophoresis as described for unlabeled oligosaccharide acceptors.
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α1 → 3-mannosyltransferase has very low activity with α1 → 2-mannotetraose (6). The reaction was dependent on protein concentration and was linear with time up to 2 h. The enzyme required Mno⁺ or Co³⁺ for activity and it was stimulated by nonionic detergents.

**Purification and Properties of the Enzyme**—Table II summarizes purification of the mannosylphosphate transferase from *S. cerevisiae* X2180 cells grown to stationary phase. Such cells bind Alcian Blue dye (18) and were presumed to possess high transferase activity. Subsequent experiments revealed that *S. cerevisiae* mnn1 cells isolated during early logarithmic growth were also a good source of the mannosylphosphate transferase, and this was the enzyme used in most of the experiments.

Several nonionic detergents, representing a range of hydrophile-lipophile balance values (25), were tested for their ability to solubilize and stimulate the transferase activity. Brij 58 and Brij 35 solubilized almost as much activity as Triton X-155 and Brij 56, but they also solubilized more protein. Triton X-155 was selected for routine use because it gave good activity and formed a stable suspension in water at 4°C. The ratio of detergent to protein was important in determining whether the detergent stimulated or inhibited the activity. A detergent to protein weight ratio of 0.05 to 0.25 gave the highest activity, 2 to 4 times that without detergent. Although a mixture of 2 M urea and Triton X-100 is suitable for solubilization of the mannosyltransferases from *S. cerevisiae* (6), urea inactivated the mannosylphosphate transferase, even under conditions in

| Oligosaccharide | Structure | Mannan source |
|----------------|----------|---------------|
| Mannobiose     | aM → ¹M | *S. cerevisiae* |
| Mannotriose-I  | aM → ²aM → ³M | *K. brevis* |
| Mannotriose-II | aM → ²aM → ³M | Saccharomyces chevalieri |
| Mannotetraose-I| aM → ²aM → ³aM → ³M | *H. polymorpha* |
| Mannotetraose-II| aM → ²aM → ³aM → ³M | *S. cerevisiae* |
| Mannopentaose-I| aM → ³aM → ³aM → ³aM → ³M | *H. polymorpha* |
| Mannopentaose-II| aM → ⁴M + aM → ³M | *S. cerevisiae* |
| Mannobiose phosphate | aM → ⁴M | *K. brevis* |
| Mannotriose-I phosphate | aM → ²aM → ³M | *K. brevis* |
| Mannotetraose-II phosphate | aM → ³aM → ²aM → ²M | *S. cerevisiae* |

* A mixture of pentasaccharides composed primarily of these fragments in a ratio of 1:5 (16). Mannohexaose and mannobetaose isomers with structures similar to those of the mannopentaose-II have also been used in this study (see Fig. 5).

**Table II**

| Purification step | Volume | Total protein | Total activity * | Specific activity * | Purification | Yield |
|------------------|--------|--------------|-----------------|--------------------|--------------|-------|
| Crude extract from 200 g of cells | 490 | 12,800 | 25.3 | 198 | 1 | 100 |
| 100,000 × g pellet | 100 | 4,010 | 7.8 | 194 | 1 | 31 |
| Washed EDTA pellet | 82 | 2,968 | 5.8 | 195 | 1 | 23 |
| Pellet plus Triton X-155 | 102 | 3,029 | 29.0 | 940 | 5 | 115 |
| Detergent extract of pellet | 88 | 2,094 | 25.0 | 1,193 | 6 | 100 |
| Ammonium sulfate fractionation (20 to 40%) | 28 | 182 | 5.0 | 2,747 | 14 | 20 |
| Bio-Gel A-1.5m column chromatography | 19 | 21 | 3.4 | 16,190 | 83 | 14 |
| Cellulose P column chromatography | 70 | 10 | 4.9 | 49,000 | 253 | 20 |

* Activity was measured without added detergent. One unit of activity catalyzes the formation of 1 pmol of product/h in the standard assay using 6 mM GDP-mannose and 10 mM reduced mannobetaose-I.

Although the differential centrifugation and KJ1A treatment often led to low yields without significant purification, as shown here, they were included because a preparation resulted that contained only the membrane-bound enzyme activity in which a metal dependency could be demonstrated. In some experiments, a recovery of 78% was observed.

The apparent increase in total units reflects stimulation by Triton X-155 in the enzyme extract. The subsequent drop in activity is due to its removal.
which the detergent alone stimulated enzyme activity.

Enzyme, solubilized by Triton X-155, was chromatographed on a Bio-Gel A-0.5m column equilibrated with buffer containing 0.5% detergent. The activity co-migrated with a single broad peak of protein that was eluted about halfway through the resolving space of the column. In the absence of detergent, activity recovered by (NH₄)₂SO₄ fractionation was excluded from Bio-Gel A-1.5m, indicating that the solubilized enzyme may aggregate when the detergent concentration is low.

Both the EDTA-treated and the detergent-solubilized enzyme exhibited maximal mannosylphosphate transferase activity from pH 6.5 to 8.5. Activity was inhibited by salt, and at 0.4 M NaCl or 0.3 M sodium phosphate, pH 7, less than 20% of the control value was observed. Full activity was restored by dialysis to remove the salt. Mn²⁺, Ca²⁺, and Mg²⁺ inhibited, and Mg²⁺, K⁺, Li⁺, and Na⁺ had no effect.

The enzyme was routinely stored in detergent. After 3 weeks, the extract in 1% Triton X-155 containing 25 mM imidazole acetate, pH 6.5, retained 85% of the activity when stored at 4°C and 60% if stored at -20°C. When incubated in the presence of substrates, the detergent-solubilized activity was stable at 30°C for 2 h. In the absence of substrates, all activity was lost within 5 min at 50°C.

**Evidence for the Phosphodiester Structure of the Assay Product**—The products formed in an incubation of solubilized enzyme, reduced [³H]mannotetraose-I, and GDP-[¹⁴C]mannose were chromatographed on a DEAE-Sephadex A-25 column, which fractionates on the basis both of size and charge. The sole doubly labeled compound was eluted in a position ahead of reduced mannotetraose-II phosphate, suggesting that it was larger and less charged than this monoester reference (data not shown). Mild acid hydrolysis of the compound, under conditions that selectively break glycosylphosphate linkages, yielded [³H]mannose and reduced [¹⁴C]mannotetraose-I phosphate (Fig. 1), showing that both labeled substrates were incorporated.

The labeled product, isolated from an enzymatic reaction with reduced [³H]mannotetraose-I and unlabeled GDP mannose, migrated as a single radioactive peak between the reduced mannotetraose-II phosphate and mannotetraose-I phosphate moeister (Fig. 2A). Although this product was unaffected by treatment with phosphomonoesterase (Fig. 2B), mild acid hydrolysis yielded a new labeled substance with the electrophoretic property of reduced mannotetraose phosphate (Fig. 2C) that was susceptible to phosphomonoesterase digestion which converted it to a neutral labeled compound with the property of the starting oligosaccharide acceptor (Fig. 2D).

The position of phosphate attachment to the oligosaccharide acceptor was established by exhaustive digestion with jack bean α-mannosidase (29). Reduced mannotriose phosphate was the smallest radioactive product obtained from the digestion of reduced [³H]mannotetraose-I phosphate, whereas similar treatment of the reference compound reduced [³H]mannotetraose-II phosphate (Fig. 3) yielded reduced manno-biose phosphate, as expected from the published structure (3). These results indicate that the mannosylphosphate unit in the product formed in the enzyme assay is attached to the mannose next to the nonreducing terminus, analogous to its location on the trisaccharide side chains in the mnn1 mutant mannans (24). The reactions employed in this characterization are summarized in Fig. 4.

**Acceptor Specificity**—Oligosaccharides (Table 1), labeled by reduction with [³H]borohydride, were tested as acceptors for mannosylphosphate transfer with the solubilized enzyme and GDP-mannose. Both the number of mannose units in the molecule and the linkage were important for activity (Table III). The reduced α1 → 2-mannotetraose was the best acceptor, although the Kₘ is apparently so high that it did not saturate the enzyme even at 50 mM concentration. A tetrasaccharide containing a terminal α1 → 3-linked mannose gave only 10% as much product.

Larger oligosaccharides, with an α1 → 6 linkage connecting two “side chain” units (2), were assayed with GDP-[¹⁴C]mannose as the donor (Fig. 5). None showed significant mannosylphosphate acceptor activity. Instead, the principal labeled product was the neutral oligosaccharide 1 mannose residue larger than the acceptor (established by gel filtration but data not shown), probably resulting from the action of an α1 → 6-mannosyltransferase in the preparation which is known to favor such branched acceptors (6). The large amount of GDP-[¹⁴C]mannose remaining at the end of the incubation, and the small amount of [¹⁴C]mannosylphosphate produced, show that the low activity of the mannosylphosphate transferase is not due to nonproductive degradation of the donor by contaminating enzymes. In fact, as much as 50% of the labeled mannose is incorporated into neutral products in the incubation with the mannohexaose, and less than 5% appears as mannosylphosphate. Exogenous mannan from the mnn1,mnn4 mutant, which should contain many potential acceptor sites, was not an acceptor and did not compete with the oligosaccharide acceptor.

**Specificity of Glycosylphosphate Donors**—GDP-mannose was the best glycosylphosphate donor although the glucose derivative did show some activity (Table IV). The apparent Kₘ for GDP mannose was about 1 mM, but the enzyme was inhibited at higher concentrations. Dolichyl [¹⁴C]mannosylphosphate, from yeast, gave no acidic radioactive product when incubated with enzyme and mannotetraose-I (Fig. 6).

Both GDP and GMP, but not guanosine, inhibited the reaction significantly, as did mannose 6-phosphate and manno-biose 6-phosphate, presumably because of their similarity to the reaction product. The mnn1 mannans, which should be analogous to the reaction product, was a poor inhibitor compared to wild type and H. polymorpha mannans.

**Mannosylphosphate Transferase in Yeast Mannan Mutants**—The mannosylphosphate transferase activities in various S. cerevisiae strains, except those containing a mnn4
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The mnn4 mutants showed much less activity. Even the heterozygous mnn4/wild type diploid had less than one-fourth the activity observed for the others, and it was comparable to the haploid mutant itself. If one-fourth of the mnn1 pellet was replaced by buffer or mnn4 pellet, 75% of the original mannosylphosphate transferase activity was observed. Thus, the mnn4 extract was inactive and did not inhibit the action of the wild type enzyme. Transferase activity in protoplasts and in detergent-solubilized preparations behaved similarly to that in crude cell extracts.

No degradation of [3H]mannotetraose-I mannosylphosphate was observed when it was incubated with the pellet fraction or the crude extract from any of the strains with or without GDP-mannose (data not shown). These results suggest that the extracts did not contain an active phosphodiesterase capable of hydrolyzing the mannosylphosphate diester group in vitro.

Isolation of New Mannosylphosphate Transferase Mutants—Cultures of S. cerevisiae XW-452 (mnn1) were mutagenized with ethyl methanesulfonate and negative selection procedures (5) were employed to obtain seven clones that did not bind Alcian Blue dye or agglutinate with anti-K. brevis, anti-mnn2, or anti-X2180 sera. Segregation of the mnn4 trait, determined by Alcian Blue dye binding and immunochemical analysis of the tetrad of matings with parental and wild type strains, showed that each mutation involved a single gene that was not linked to mnn1. The diploids of three of the clones crossed with mnn1 did not bind dye; thus, these mutations, like the original mnn4 strain, were dominant. Four of the isolates were recessive, since the heterozygous diploid formed with the mnn1 strain exhibited the properties of mnn1 cells. All of the recessive mutants were found to be allelic by complementation tests in the diploid. Although they gave the acetolysis pattern characteristic of mnn4 mutants (Fig. 7), lacking only the peak for mannotetraose phosphate, they segregated independently of the dominant mnn4 strain (4 parental ditypes, 4 nonparental ditypes and 15 tetratypes in 23 ascii dissected). The results suggest that they involve a new genetic locus for mannosylphosphate transferase activity.

The new locus is designated mnn6, and its properties are compared to those of other strains in Table V.

**DISCUSSION**

Early investigations of yeast mannan biosynthesis examined the incorporation of labeled mannose (28–30) and mannosylphosphate groups (31, 32) from GDP-mannose into endogenous polymers. Bretthauer et al. (32), in particular, provided the first clear demonstration for the existence of the latter reaction. Although these experiments revealed some important features of the process, the complexity of the mannan molecule precluded a definition of the individual steps. Recently, the characterization of mannans from yeast strains in which the cell surface phenotype is altered by mutagenesis has led to the postulation of a detailed pathway for yeast mannoprotein biosynthesis in Saccharomyces (4, 27), and selective assays have been developed for studies on biosynthesis of the serine- and threonine-linked sugars (22), the asparagine-linked core sugars (33), and the outer chain sugars (6, 34). The focus of this study is the presumed mannosylphosphate transferase that is involved in the incorporation of phosphate into the mannan molecule. Although mannosylphosphate transfer in S. cerevisiae has not been reported
previously, a number of mutants with phosphate-deficient mannans were known (5, 7). Because of the dominance of one of these mutations (35), we felt that a detailed study of the enzyme that catalyzes this reaction could provide new insight into the mechanisms by which mannoprotein biosynthesis is regulated.

The assay procedure we have developed is convenient because the neutral oligosaccharide acceptor is labeled rather than the nucleotide sugar donor. Thus, neither the charged degradation products, which are unlabeled, nor the neutral labeled by-products complicate the measurement of radioactive material converted to an acidic form by enzymic action. The use of an exogenous acceptor has made it possible to demonstrate that the mnn2 mutant, which was presumed to

FIG. 3. Localization of the phosphate group on reduced [3H]mannotetraose-I phosphate. A, electrophoresis of the [3H]-oligosaccharide phosphate produced by exhaustive digestion with α-mannosidase. B, paper chromatography of the neutral oligosaccharides obtained by phosphomonoesterase treatment of the α-mannosidase-digested [3H]-oligosaccharide phosphates. Closed symbols represent the reference compounds, open circles are for [3H]mannotetraose-I phosphate, and open triangles are for [3H]mannotetraose-II phosphate. Refer to Table I for exact structures.

FIG. 4. Scheme for characterization of the mannosylphosphate transferase assay product. The enzyme-catalyzed reaction (a) yields a product that is stable to phosphomonoesterase, but it is hydrolyzed by mild acid (b) to give a product that is converted by the phosphatase (d) to a neutral oligosaccharide. The same product is converted by α-mannosidase (c) to a mannotriose phosphate that is degraded by phosphatase (d) to a neutral trisaccharide. * indicates position of [3H].
have a phosphate-deficient mannan because it lacked endogenous acceptors (27), does possess a normal level of mannosylphosphate transferase activity.

The solubilized enzyme has properties that are consistent with a role in mannan biosynthesis; it has a preference for acceptor oligosaccharides with an α1 → 2 linkage at the nonreducing end of the chain, and it has a much lower activity with acceptors that have been modified by the α1 → 3-mannosyltransferase. Moreover, the structure of the product synthesized in vitro is analogous to that of phosphorylated side chains in mannan from a mnn1 mutant.

The acceptor structural specificity for the mannosylphosphate transferase in S. cerevisiae differs from that for the N-acetylglucosamine transferase in Kluyveromyces lactis (36). The latter enzyme shows a specificity for an α1 → 3 nonreducing terminus, and the mnn1 mutant of K. lactis, that is defective in an α1 → 3-mannosyltransferase, lacks N-acetylglucosamine because the acceptor is missing. This situation is analogous to the phosphate-deficient mannan found in mnn2 and mnn5 strains of S. cerevisiae, in which the mannosylphosphate acceptor site is absent due to defective α1 → 2-mannosyltransferases. In each of these mutants, the respective transferase activities are found in extracts when assayed with exogenous acceptors.

Although the relative specificity of the enzyme is apparent, mannosylphosphate transfer to reduced mannotetraose-I is about 200-fold slower than the rate calculated for the corresponding activity in the cell. The Kₐ for the reduced tetrasaccharide acceptor appears to be greater than 1 mM, which is far higher than the 0.2 to 7.5 mM values reported for various mannosyltransferase acceptors (6), 13 mM for mannotetraose in N-acetylglucosamine transfer (39), or even 100 mM for mannosylphosphate as an acceptor for mannobiose synthesis (33). On the other hand, the Kₐ observed for GDP-mannose as a mannosylphosphate donor is comparable to that determined for manno transfer to exogenous acceptors by Nakajima and Ballou (6) and to endogenous acceptors in Saccharomyces carlsbergensis by Behrens and Cabib (28) and by Mayer (31) for Hansenula phosphomannan. Exogenous mannan from mnn1, mnn4 and mnn1 mutants, which would be expected to have acceptor and product sites, did not compete significantly with the reduced mannotetraose. Thus, we found no evidence that the enzyme can act on large mannan molecules.

No evidence was obtained that a lipid-bound mannosylphosphate derivative was involved in the reaction. Labeled oligosaccharide phosphate was not detected in incubations in

**Table IV**

| Glycosylphosphate donor specificity | Relative activity |
|-----------------------------------|------------------|
| GDP-mannose                       | 100              |
| ADP-mannose                       | 32               |
| GDP-glucose                       | 20               |
| UDP-glucose                       | 1                |
| ATP, GTP, GDP, GMP                | 0                |
| Mannose 1-phosphate, mannose 6-phosphate | 0              |

**Table V**

| Genotype | Yeast strain | Man/P ratio in mannan | Alcian Blue dye binding in cells | Relative transferase activity |
|----------|--------------|-----------------------|---------------------------------|------------------------------|
| mnn1     | LB1-22D      | 20 (28)               | ++                              | 100                          |
| Wild type| X-2180       | 30 (48)               | +                               | 85                           |
| mnn2     | LB1-16A      | 300 (60)              | -                               | 71                           |
| mnn5     | LB6-5A       | 355 (71)              | -                               | 97                           |
| mnn4     | LB6-5D       | 300 (60)              | -                               | 17                           |
| mnn1, mnn4| LB6-16C     | (230)                 | -                               | 16                           |
| +/mnn4   | LB6-5D × LB1-22D | 102          | -                               | 20                           |
| mnn6     | LB94-IC      | 137 (27)              | +                               | 19                           |
| +/mnn6   | LB94        | 50 (10)               | +                               | 89                           |

* Data in parentheses are from Ballou et al. (7).
+ Compared to the mnn1 mutant extract.
× Derived from strain X2180-1A4 (6).
° Derived from strain X2180-1A5 (5).
# Derived from strain 712 (R. Cohen and L. Ballou, unpublished). This mutant has properties similar to strain X2180-1B2 which is affected in the mnn3 locus (5). The mnn5 mutant also makes mannan with shortened, unphosphorylated side chains.
+ Derived from strain 4484-24D-1 (7).

**FIG. 6.** Test of dolichyl mannosylphosphate as a potential donor. Electrophoretic analysis of the water-soluble products from incubation of the mannosylphosphate transferase with mannotetraose-I and dolichyl [14C]mannosylphosphate (bottom) or GDP-[14C]mannose (top). The dolichyl mannosylphosphate remains at the origin in this system.
which dolichyl mannosylphosphate was present as a potential donor, whereas much of the labeled mannose was incorporated into apparently neutral products. The result is not unexpected because thermodynamic considerations predict the involvement of a mannosylpyrophosphate derivative as the donor for a mannosylphosphate group, and such a derivative of dolichol has not been detected in yeast.

The dominance of the mnn4 mutation, which leads to a reduced level of mannosylphosphate groups even in the mannan of the mnn4/wild type heterozygous diploid (7, 95), remains an unexplained phenomenon. Lowered phosphate levels in the mannan of the heterozygote could arise in two ways—either the wild type allele is expressed but cannot compensate for the mutant allele or the mutant allele blocks all wild type expression. Mechanisms consistent with the first explanation could involve the production of an inhibitor of the transferase or an enzyme that degraded the product. We found no support for either of these possibilities; no inhibitor specific to the mnn4 strain was detected by mixing extracts, nor could we demonstrate a phosphodiesterase that cleaved the mannosylphosphate mannotetraose product. An additional possibility could involve the production of an inhibitor of the transferase that prevents equilibration of subunits under the conditions of our assay. However, extracts from the heterozygous diploid, in which mixing may occur in vivo, do not show significantly more mannosylphosphate transferase activity.

Thus, we are led to consider an explanation of the second type, namely that the mnn4 mutant allele may block expression of the wild type allele by preventing transcription of both copies of the structural gene in the heterozygous diploid. Although we have no direct support for this hypothesis, we have obtained another class of mannosylphosphate transferase mutants, wherein designated mnn6, with the recessive property expected of a structural gene mutation. The mnn6 mutants have the dye binding phenotype of the mnn4 class and they synthesize mannan altered only by the absence of the mannosylphosphate group, yet this locus segregates independently of mnn4 thus implying that the latter has a regulatory role.

Acknowledgment—We thank Lun Ballou for constructing some of the yeast strains used in this work, for determining some of the segregation patterns, and for checking the mannose/phosphate ratios of the mannans.

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