Effects of Nucleotides and Sugar Nucleotides on Mannosyltransferase Activity in *Saccharomyces cerevisiae*

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(Received 28 November 1980; revised 19 January 1981)

A survey was made of the effects of different nucleotides, sugar nucleotides and sugars on mannosyltransferase activity in *Saccharomyces cerevisiae* to assess their possible role in the regulation of mannan synthesis. Mannosyltransferase activity in spheroplast lysate and washed membrane preparations was not markedly affected by sugars, nucleoside monophosphates or by most of the sugar nucleotides tested. GDP and GTP both inhibited enzyme activity. ATP caused a significant stimulation, and analysis of the mannan formed suggested that this was due mainly to increased synthesis of the polysaccharide moiety. GDPglucose was a competitive inhibitor of total mannan synthesis and also inhibited the formation of mannanolipids.

INTRODUCTION

The major components of the cell wall of *Saccharomyces cerevisiae* are mannan (Ballou, 1976) and three β -glucans (Manners *et al.*, 1973*a, b*; Fleet & Manners, 1976). Mannan is present as a mixture of mannoproteins in which the carbohydrate moieties comprise short alkali-labile oligosaccharides attached to serine or threonine residues, and long polysaccharide chains linked via di-*N*-acetylchitobiose to asparagine (Ballou, 1976). Biosynthesis of the carbohydrate chains probably involves at least ten different mannosyltransferases, each one catalysing the formation of a specific glycosidic linkage (Nakajima & Ballou, 1975). The mannosyl residues all originate from GDPmannose (Behrens & Cabib, 1968) either by direct transfer or via lipid intermediates which have been identified as dolichyl phosphate derivatives (Jung & Tanner, 1973). β -Glucans have been synthesized in vitro from UDP-glucose (Sentandreu *et al.*, 1975; López-Romero & Ruiz-Herrera, 1977; Shematek *et al.*, 1980) and from GDPglucose (Bálint *et al.*, 1976).

Relatively little is known about the regulation of glucan and mannan biosynthesis. Shematek & Cabib (1980) have shown that 1,3- β -glucan synthase activity is stimulated by ATP or GTP. Similarly, Elorza *et al.* (1977) found that mannosyltransferase activity was inhibited by nucleoside diphosphates and suggested that rapid modulation of the rate of mannoprotein formation might be effected by changes in the nucleotide pool. We have recently reported a further mechanism involved in regulation of mannan synthesis in which mannosyltransferase activity is suppressed by a heat-labile, trypsin-sensitive inhibitor (Harrington & Douglas, 1980). In the present study we have surveyed the effects of various nucleotides and sugar nucleotides on mannan synthesis in vitro and now report that mannosyltransferase activity is competitively inhibited by GDPglucose but enhanced by ATP.

METHODS

Organisms and growth conditions. *Saccharomyces cerevisiae* X2180-1A (a) and its mutants defective in mannoprotein biosynthesis, LB1-3B *mnn2-1a* and LB1-10B *mnn1-2a*, were kindly supplied by Dr C. E. Ballou, University of California, Berkeley, U.S.A. They were grown aerobically, as described by Harrington & Douglas (1980).

Preparation of spheroplast lysate and washed membrane fraction. These were prepared as described previously (Harrington & Douglas, 1980) except that Zymolyase-5000 was used at a concentration of 0.7 mg ml^{-1} and incubation was for 60 min at 30°C .

Mannosyltransferase assay. Mannosyltransferase activity was measured by following the incorporation of [^{14}C]mannose from GDP[^{14}C]mannose into perchloric acid-precipitable material as described by Harrington & Douglas (1980). The standard incubation mixture contained spheroplast lysate or washed membrane fraction at a protein concentration of 1 mg ml^{-1} . The filtration procedure was modified slightly in that filters were prewashed with cold 1% (w/v) mannose solution and precipitates on filters were washed with cold 0.3 M-HClO_4 (20 ml) followed by more cold 1% mannose solution (5 ml). This assay method measures the formation of both glycoproteins and glycolipids containing [^{14}C]mannose since these products co-precipitate on the filters.

β -Elimination of [^{14}C]mannoproteins. Following incubation, mannosyltransferase assay mixtures containing washed membrane fraction (0.2 mg protein) were heated at 100°C for 3 min, adjusted to 1 ml with water and centrifuged (1000 g, 5 min). The pellets were washed twice with water, resuspended in 0.1 M-NaOH (1 ml) and incubated at room temperature ($18\text{--}20^\circ\text{C}$) for 18 h. After neutralization with 2 M-acetic acid (50 μl), the mixtures were centrifuged and the pellets washed twice with water. The pellets were finally transferred to scintillation vials with methanol (1.5 ml). Methanol was removed by evaporation *in vacuo* and radioactivity was counted in toluene scintillant (10 ml). Control mixtures were incubated for 18 h with water instead of alkali and zero-time controls were from assay mixtures containing heat-inactivated (100°C for 3 min) washed membrane fraction.

Radioactivity incorporated into lipids. Radioactive lipids were extracted from standard mannosyltransferase assay mixtures by two successive extractions with different chloroform/methanol mixtures. Assays were terminated by the addition of ice-cold chloroform/methanol (2:1, v/v; 2 ml) followed by thorough mixing. With zero-time controls, chloroform/methanol was added before GDP[^{14}C]mannose. Each mixture was centrifuged at 2000 g for 10 min and the pellet was extracted twice with 2 ml of the same solvent. KCl (0.88%, w/v; 1.5 ml) was added to the combined extracts (6 ml) and the resultant lower phase was washed twice with theoretical upper phase (1.5 ml) according to Folch *et al.* (1957). The washed lower phase was transferred to a scintillation vial, evaporated to dryness *in vacuo* and counted in toluene scintillant (10 ml). The residual pellet was extracted twice with chloroform/methanol/water (10:10:3, by vol.; 2 ml) and the combined extracts were dried and counted. Individual lipids were separated by thin-layer chromatography of concentrated extracts (50 μl) on plates of silica gel G (Merck) in chloroform/methanol/water (65:25:4, by vol.).

Degradation of sugar nucleotides. Degradation of ^{14}C -labelled sugar nucleotides by enzyme preparations was assessed by separating the reaction products by paper chromatography. After incubation of mannosyltransferase assay mixtures, or mixtures containing equivalent concentrations of GDP[^{14}C]glucose or UDP[^{14}C]glucose (Amersham) in place of GDP[^{14}C]mannose, reactions were terminated by heating at 100°C for 3 min. The mixtures were applied as 2.5 cm bands to Whatman 3MM paper and subjected to descending chromatography. Following development, the radioactivity on each track was located by counting 1 cm segments in toluene scintillant (5 ml). Radioactive components which neither remained at the origin nor co-migrated with sugar nucleotide standards were regarded as products of sugar nucleotide hydrolysis. Percentage degradation refers to the combined radioactivity in these products expressed as a percentage of the total radioactivity on the chromatogram.

Paper chromatography. This was carried out on Whatman 3MM paper with the descending solvent system isobutyric acid/1 M aqueous ammonia (5:3, v/v). Unlabelled standard sugars were detected with alkaline silver nitrate (Trevelyan *et al.*, 1950) and phosphate esters with perchloric acid/molybdate (Hanes & Isherwood, 1949). Sugar nucleotides were visualized under ultraviolet light.

Protein determinations. Protein was estimated by the Lowry method using bovine plasma albumin as a standard. Before analysis, samples of spheroplast lysate and washed membrane fraction were digested in 0.5 M-NaOH at 100°C for 15 min in glass-stoppered tubes.

RESULTS

Survey of effectors of mannosyltransferase activity

Mannosyltransferase activity of both spheroplast lysate and washed membrane preparations was assayed in the presence of a variety of possible effectors, all at a final concentration

Table 1. *Effect of sugars and nucleotides on mannosyltransferase activity of S. cerevisiae*

Addition to incubation mixture (1 mM)	Relative enzyme activity (%)*	
	Spheroplast lysate preparation	Washed membrane fraction
None	100.0	100.0
Glucose	101.2 ± 1.8	102.5 ± 1.0
Mannose	96.3 ± 5.7	89.4 ± 3.4
<i>N</i> -Acetylglucosamine	97.2 ± 1.0	99.4 ± 0.5
Glucosamine	100.4 ± 6.0	101.8 ± 3.6
Cyclic AMP	98.6 ± 3.9	96.0 ± 2.2
AMP	97.7 ± 0.7	97.1 ± 1.4
ADP	119.3 ± 5.3	114.1 ± 2.1
ATP	242.4 ± 6.3	232.4 ± 7.2
GMP	94.9 ± 3.1	100.0 ± 4.4
GDP	38.9 ± 3.2	40.3 ± 0.3
GTP	65.0 ± 3.4	66.3 ± 2.6
UMP	88.9 ± 4.2	105.1 ± 3.3
UDP	101.1 ± 3.8	97.1 ± 1.4
UTP	73.3 ± 5.5	120.5 ± 2.3
P _i	106.3 ± 6.0	120.3 ± 3.4
PP _i	100.0 ± 5.4	120.9 ± 5.4

* Activity is expressed as a percentage of that in standard incubation mixtures with no additions. Values quoted are means of at least three determinations, ± the standard deviation.

Table 2. *Effect of sugar nucleotides on mannosyltransferase activity of S. cerevisiae*

Addition to incubation mixture (1 mM)	Relative enzyme activity (%)*	
	Spheroplast lysate preparation	Washed membrane fraction
None	100.0	100.0
UDP- <i>N</i> -acetylglucosamine	101.0 ± 1.9	101.7 ± 3.0
ADPglucose	112.1 ± 1.5	100.2 ± 1.3
CDPglucose	103.4 ± 4.8	91.5 ± 1.6
dTDPglucose	99.5 ± 3.1	94.1 ± 2.4
UDPglucose	95.2 ± 5.6	104.1 ± 4.6
GDPglucose	19.4 ± 5.0	23.8 ± 7.0
UDPglucose + GDPglucose	19.4 ± 4.7	24.9 ± 7.0
UDPmannose	80.1 ± 5.9	79.4 ± 4.9
ADPmannose	75.7 ± 4.1	89.0 ± 3.6

* Activity is expressed as a percentage of that in standard incubation mixtures with no additions. Values quoted are means of three determinations, ± the standard deviation.

of 1 mM (Tables 1 and 2). The sugars and nucleoside monophosphates tested had little effect on enzyme activity (Table 1). ATP, however, stimulated activity in both preparations by more than 130%. There was also slight stimulation by ADP while GTP and, more especially, GDP inhibited activity. UTP inhibited activity of the spheroplast lysate but stimulated that of the washed membrane fraction.

Only three of the eight sugar nucleotides tested affected mannosyltransferase activity by more than 12% (Table 2). ADPmannose and UDPmannose inhibited activity of both enzyme preparations by 11–24%. GDPglucose, on the other hand, caused 81% and 76% inhibition with the spheroplast lysate and washed membrane preparations, respectively. When GDPglucose and UDPglucose were both included in reaction mixtures, inhibition was the same as that due to GDPglucose alone. Four sugar nucleotides were further tested over a range of concentrations up to 2 mM. GDPglucose was a potent inhibitor of mannosyltransferase activity, causing almost complete inhibition at 2 mM. Inhibition by ADPmannose

Table 3. *Effect of ATP on mannosyltransferase activity of washed membrane fractions from wild-type and mutant strains of S. cerevisiae*

Strain of <i>S. cerevisiae</i>	Relative enzyme activity (%) [*] with 5 mM-ATP	
	Total mannan synthesis	Non β -eliminable synthesis
X2180-1A (wild-type)	172	791
<i>mnn2</i> mutant	179	213
<i>mnn1</i> mutant	142	132

^{*} Activity is expressed as a percentage of that without ATP.

and UDPmannose was maximal at about 1 mM while UDP-*N*-acetylglucosamine had little effect on enzyme activity at any of the concentrations tested.

Stimulation of mannosyltransferase activity by ATP

The formation of dolichyl monophosphate mannose from GDPmannose by liver microsomes is inhibited by rapid hydrolysis of the substrate by nucleotide pyrophosphatase but substrate degradation can be prevented by ATP (Vessey & Zakim, 1975). In the present study, stimulation of mannosyltransferase activity by ATP was not due to the protection of GDP[¹⁴C]mannose from pyrophosphatase degradation. Paper chromatography of standard reaction mixtures containing 100 μ g of spheroplast lysate protein revealed that less than 1% of the substrate was hydrolysed to mannose 1-phosphate or mannose during incubation.

The short oligosaccharides attached to serine or threonine residues in yeast mannoproteins are released by β -elimination on treatment with mild alkali (Sentandreu & Northcote, 1968). This technique was used to analyse the nature of mannan synthesized *in vitro*. ATP enhanced synthesis of the non β -eliminable portion of mannan much more than synthesis of total mannan with enzyme preparations from the wild-type (Table 3). These results may indicate a preferential stimulation of polysaccharide chain synthesis. With enzyme preparations from two mutant strains unable to form complete polysaccharide chains (Ballou, 1976), ATP enhanced synthesis of total mannan and non β -eliminable mannan almost equally.

The extent to which mannan synthesis was stimulated by ATP varied with different enzyme preparations. Furthermore, ATP-mediated activation tended to diminish following storage of enzyme preparations at -20°C , although it could sometimes be restored at higher ATP concentrations. Since it is known that some mannosyltransferase activities are lost more rapidly than others during storage at low temperatures (Sharma *et al.*, 1974; Parodi, 1979) these observations may also imply selective stimulation of some transferases by ATP.

Inhibition of mannosyltransferase activity by GDPglucose

Mannosyltransferase activity of the spheroplast lysate preparation was measured at different substrate concentrations. A Lineweaver-Burk plot of the results (Fig. 1) revealed biphasic kinetics with two K_m values (0.13 mM and 0.53 mM) and two V values [6.94 and 11.43 nmol h⁻¹ (mg protein)⁻¹] for low and high substrate concentrations, respectively. Similar biphasic kinetics have been described recently by Guttierrez & Ruiz-Herrera (1979) for mannosyltransferase activity in membrane fractions from *Mucor rouxii*. In the presence of 1 mM-GDPglucose the V values remained unaltered while the apparent K_m values increased to 0.23 mM and 0.63 mM. These results indicate that GDPglucose is a competitive inhibitor of mannosyltransferases. This conclusion is supported by the data in Fig. 2 which show that inhibition of mannan synthesis by GDPglucose becomes obvious only at GDPglucose:GDP-mannose ratios of 2:1, or greater.

Fig. 1. Lineweaver-Burk plot of specific mannosyltransferase activity of a spheroplast lysate preparation against GDPmannose concentration with (O) or without (●) 1 mM-GDPglucose. Mannosyltransferase activity was measured in the standard assay with GDP[¹⁴C]mannose (578 pmol; 100 nCi) and carrier GDPmannose (0.2–2.0 mM).

Fig. 2. Inhibition of mannosyltransferase activity of a spheroplast lysate preparation by GDPglucose at different concentrations of GDPmannose. Mannosyltransferase activity was measured in the standard assay with 1 mM-GDPglucose, GDP[¹⁴C]mannose (578 pmol; 100 nCi) and carrier GDPmannose (0.0–2.0 mM). Activity is expressed as a percentage of that in the standard incubation mixture without GDPglucose.

GDPglucose inhibited the formation of mannose-containing lipids. When spheroplast lysate (0.1 mg protein) was incubated with GDP[¹⁴C]mannose under standard conditions and then extracted with two chloroform/methanol mixtures (chloroform/methanol, 2:1, v/v; followed by chloroform/methanol/water, 10:10:3, by vol.) radioactive label equivalent to 27 pmol [¹⁴C]mannose h⁻¹ (mg protein)⁻¹ was found in the combined extracts. The lipids were not fully characterized but, from their solubilities in organic solvents and *R_F* values on thin-layer chromatography, appeared to comprise a mixture of dolichyl monophosphate mannose (Tanner, 1969; Sentandreu & Lampen, 1971) and dolichyl diphosphate-bound oligosaccharides (Lehle & Tanner, 1975, 1978). When 1 mM-GDPglucose was included in incubation mixtures there was no detectable radioactive label in chloroform/methanol extracts i.e. less than 3 pmol h⁻¹ (mg protein)⁻¹.

GDPglucose glucohydrolase activity

If GDP[^{14}C]mannose was replaced in assay mixtures by an equivalent concentration (116 pmol; 20 nCi) of GDP[^{14}C]glucose, no radioactivity was found in lipid extracts. This suggested that although GDPglucose inhibits the formation of manolipids, it does not do so by competing with GDPmannose for the available dolichyl phosphate. Moreover, no detectable radioactive label was incorporated from GDP[^{14}C]glucose into perchloric acid-precipitable material under these conditions. However, when the products of reaction mixtures containing spheroplast lysate and GDP[^{14}C]glucose were separated by paper chromatography it was found that the sugar nucleotide had been largely degraded to [^{14}C]glucose. This was probably due to the action of GDPglucose glucohydrolase, a soluble enzyme, first reported in *S. cerevisiae* by Sonnino *et al.* (1966), which catalyses the hydrolysis of GDPglucose to GDP and glucose. Degradation was rapid and specific. Only 1% of GDP[^{14}C]mannose (116 pmol; 20 nCi) was hydrolysed during incubation with spheroplast lysate protein (2 mg ml $^{-1}$) for 20 min at 30 °C. Similar incubation of GDP[^{14}C]glucose and UDP[^{14}C]glucose (116 pmol; 20 nCi) with spheroplast lysate (4 mg ml $^{-1}$) resulted in 85.7% and 9.5% degradation, respectively.

Although carrier-free GDP[^{14}C]glucose was rapidly hydrolysed by spheroplast lysate preparations, less than 1% of the sugar nucleotide was converted to glucose at the concentration (1 mM) used to test its action on mannosyltransferase activity. Thus, the inhibitory effect of GDPglucose on mannan synthesis is not simply due to the production of GDP by the glucohydrolase. This conclusion is supported by the data in Tables 1 and 2 which show that GDPglucose inhibits mannan synthesis by 80% whereas GDP causes only 60% inhibition and glucose has no effect.

The extent of GDP[^{14}C]glucose degradation in standard assay mixtures was greater with spheroplast lysate (up to 93% degradation) than with washed membrane fraction (57%). Degradation was reduced to 17.4% by washing the membrane fraction a further four times in 50 mM-Tris/HCl buffer (pH 7.2). This more extensive washing also promoted the incorporation of radioactivity from GDP[^{14}C]glucose into perchloric acid-precipitable material to 35.7 pmol h $^{-1}$ (mg protein) $^{-1}$.

DISCUSSION

Of the various nucleotides tested, GDP, GTP and ATP had the greatest effect on mannosyltransferase activity (Table 1). Inhibition by GDP is to be expected since it is a product of the transferase reaction. Furthermore, GDP, and GTP in the presence of phosphatases, are known to reverse the formation of dolichyl monophosphate mannose by liver preparations (Richards & Hemming, 1972) and by membrane fractions from *S. cerevisiae* (Tanner *et al.*, 1971).

The mechanism by which ATP stimulates mannosyltransferase activity is less clear. The results in Table 3 suggest a preferential effect on the synthesis of mannoprotein polysaccharide chains. Related observations have been made in studies on glycoprotein synthesis by mammalian systems. For example, various nucleotides, including ATP, have been found to diminish the synthesis of dolichyl monophosphate mannose while simultaneously enhancing the formation of lipid-bound oligosaccharides by retina or rat-liver preparations (Kean, 1977; Richard *et al.*, 1978). Recently, ATP and GTP have been shown to stimulate yeast 1,3- β -glucan synthase which appears to be located on the inner surface of the plasma membrane (Shematek *et al.*, 1980; Shematek & Cabib, 1980). The characteristics of activation by the two nucleotides differed in that activation by ATP was time- and temperature-dependent and required the presence of a heat-stable, dialysable supernatant factor. On the basis of their results, Shematek & Cabib (1980) have proposed a model for nucleotide-mediated regulation of glucan synthase activity.

While UDPglucose is known to be a precursor for 1,3- β -linked glucan in yeast (Sentandreu *et al.*, 1975; López-Romero & Ruiz-Herrera, 1977; Shematek *et al.*, 1980) the role of GDPglucose in glucan synthesis is not fully established. Bálint *et al.* (1976) have reported the incorporation of low levels of [^{14}C]glucose from GDP[^{14}C]glucose into a product which they tentatively characterized as a 1,6- β -glucan containing some 1,3- β -linkages in the side-chains. Thus, GDPglucose may function as a precursor for the minor 1,6- β -glucan component of the yeast wall. Further evidence might be obtained from studies with enzyme preparations devoid of GDPglucose glucohydrolase activity. In the present investigation, extensive washing of membrane fractions decreased this activity but did not remove it entirely. If, indeed, GDPglucose is a precursor for glucan synthesis, our finding that it is also a competitive inhibitor of mannan synthesis would suggest that the two synthetic systems are strictly segregated *in vivo*. Moreover, the presence of a hydrolase with strict specificity for GDPglucose implies the existence of complex regulatory mechanisms.

This work was supported by a Science Research Council studentship awarded to C. R. H.

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