BIOSYNTHESIS OF YEAST MANNAN: INHIBITION OF SYNTHESIS OF MANNOSE ACCEPTOR BY CYCLOHEXIMIDE*

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1. Introduction

Yeast protoplasts fail to regenerate a complete new wall when they are incubated in the presence of cycloheximide [1, 2], an antibiotic known to inhibit protein synthesis at the ribosomal level [3, 4]. Sentandreu and Northcote [5] noted that incorporation of threonine into the wall of *Saccharomyces cerevisiae* was halted by the antibiotic, whereas incorporation of radioactivity from ^{14}C-glucose was only partially inhibited. This partial inhibition reflects the fact that cycloheximide stops the synthesis of the mannan of the cell wall, but does not affect the formation of glucan, the other main type of polymer [6]. The mannan polymers have been found covalently bound to peptides, either as structural [7] or as enzymatically active macromolecules [8]. In view of this, it is probable that mannan (but not glucan) synthesis requires the addition of sugar units to a polypeptide whose formation is sensitive to cycloheximide.

The findings of the present investigation are that cycloheximide inhibits protein and mannan synthesis in *S. cerevisiae* to a similar extent, and this inhibition results in an accumulation of GDP-mannose. It has no effect on the incorporation of mannose from GDP-mannose into endogenous acceptor(s) by a particulate preparation. It is concluded that cycloheximide inhibits mannan synthesis by preventing the formation of the polypeptide acceptor.

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2. Results

The degree of inhibition of protein and mannan synthesis in *S. cerevisiae* LK2G12 as a function of cycloheximide concentration is illustrated in table 1. The incorporation of ^{14}C-threonine into trichloroacetic acid precipitable material and of ^{14}C-glucose into mannan were both inhibited approximately 40% at about 0.2 μg per ml, and more than 90% at high antibiotic level (5 μg per ml). Glucan synthesis was not prevented even at 5 μg cycloheximide per ml. The similarity between the degrees of inhibition of protein and of mannan synthesis suggests that mannan synthesis is inhibited because polypeptide acceptor formation is stopped.

The effect of cycloheximide on nucleic acid and protein synthesis in yeast is reversed by washing the cells to remove the antibiotic [9]. Inhibition of mannan synthesis in *S. cerevisiae* LK2G12 could also be reversed by washing. After 30 min incubation in the presence of cycloheximide, during which time mannan was not synthesized, the cells were washed to remove the antibiotic. Incorporation of radioactivity from ^{14}C-glucose into the mannan polymers promptly resumed at a rate similar to that of control cells.

Further evidence that the inhibition of mannan synthesis parallels the effect of cycloheximide on polypeptide formation was obtained by examining wall formation in another strain of *S. cerevisiae*, in a *Saccharomyces* hybrid and in *Saccharomyces fragilis*. This last yeast is a species whose growth and protein synthesis are resistant to the antibiotic [10]. In *S. fragilis*, formation of both mannan and glucan was

Table 1
Effect of cycloheximide concentration on protein and polysaccharide synthesis in *S. cerevisiae* LK2G12.

Cycloheximide (μg/ml)	Radioactivity incorporated from ^{14}C-Glucose				^{14}C-Threonine	
	Glucan		Mannan		Protein	
	(cpm/mg cells)	Inhibition (%)	(cpm/mg cells)	Inhibition (%)	(cpm/mg cells)	Inhibition (%)
0	886	0	1005	0	48202	0
0.1	810	8	706	30	39334	20
0.2	907	−2	632	37	27060	44
0.5	925	−4	433	57	15650	78
1.0	804	9	212	79	5434	89
2.0	770	13	133	87	–	–
5.0	820	7	88	91	2666	95

Cells (4 mg) incubated in Winge medium (yeast extract, 3 g; glucose, 20 g; water, 1 litre) and cycloheximide at 30° for 10 min were supplemented with 1 μCi ^{14}C-glucose (specific activity 3.1 mCi/mmole). The cells were collected after 30 min and wall polymers extracted by the method of Northcote and Horne [22]. When ^{14}C-threonine was used, cells (0.5 mg) were supplemented with 100 nCi radioactive threonine (specific activity, 10 mCi/mmole) and subsequently treated with 5% trichloroacetic acid at 0° for 30 min and the residue collected on glass filter discs. Radioactivity was measured in a liquid scintillation spectrometer [5].

unaffected by 4 μg/ml of cycloheximide. In contrast, the *S. cerevisiae* strains and the hybrid all showed a dramatic reduction of incorporation into the mannan fractions with no significant change in glucan formation (table 2).

Siegel and Sisler [11] have reported that *Saccharomyces pastorianus* cells treated with cycloheximide showed an overall accumulation of soluble nucleotides of 116% with the mono- (216%), di- (30%) and tri- (332%) phosphates of guanosine showing the greatest increases (sugar nucleotides were not measured). We have examined the radioactive sugar nucleotides ex-

Table 2
Effect of cycloheximide on wall synthesis by *Saccharomyces* strains.

	Glucan*		Mannan*	
	Control	Cycloheximide	Control	Cycloheximide
Saccharomyces hybrid 303-67	12874	13365	3922	1076
S. cerevisiae LK2G12	1788	1926	881	75
S. cerevisiae C 1968 (6)	1549	2138	1093	159
S. fragilis	11389	12036	6775	7234

Cells were incubated in Winge medium for 10 min at 30° in the presence or absence of cycloheximide (4 μg/ml), then supplemented with 10 μCi ^{14}C-glucose for an additional 30 min. Wall polymers (glucan and mannan) were extracted as described by Northcote and Horne [22].
* cpm/mg of cells.

Table 3
Effect of cycloheximide on ^{14}C-labelled sugar nucleotides in *S. cerevisiae* LK2G12.

Nucleotide	Treatment		% of control
	Control*	Cycloheximide*	
UDP-glucose	121	220	181
GDP-mannose	70	1480	2110
UDP-NAGA	561	1160	207
TDP-glucose	870	1113	128

Two lots of cells (160 mg each) were resuspended in 200 ml medium (glucose 0.5 g; yeast extract, 3 g; bacto-peptone, 1 g; KM_2PO_4, 5 g and $MgSO_4.7H_2O$, 0.5 g; water 1 litre) and incubated at 30° for 20 min in the presence (4 μg/ml) or absence of cycloheximide. ^{14}C-Glucose (100 μCi) was then added to each suspension and after an additional 30 min the cells were washed and extracted as described by Anderson et al. [23]. Samples were applied to Whatmann no. 3 MM paper and the chromatogram developed in isobutyric acid–1 M NH_4OH (5:3). Radioactivity showed the presence of several labeled spots, only three of which received further attention (sugar nucleotides). The slowest running spot chromatographed with GDP-mannose and UDP-glucose, while the other two spots had the mobilities of UDP-NAGA and TDP-glucose. The radioactive spots were eluted with water and rechromatographed in ethanol–ethyl methyl ketone–morpholinium borate, which allows resolution of GDP-mannose and UDP-glucose [24].

* cpm/mg cells.

tracted from cells grown in the presence or absence of cycloheximide in a medium supplemented with ^{14}C-glucose. The levels of UDP-glucose and TDP-glucose, the glucosyl donors for yeast glycogen and wall glucan respectively [12], increased less than two-fold in the presence of cycloheximide (table 3). This would be expected if the transfer of their glycosyl moieties to the glucose polymers is not affected by the antibiotic. The concentration of UDP-*N*-acetylglucosamine (UDP-NAGA) was a little more elevated, perhaps because it is the source of the *N*-acetylglucosamine present in carbohydrate–protein linkages of mannan [7] and invertase [13]. In contrast, the level of GDP-mannose rose 20-fold (this is a minimum value since accumulation would have taken place during the earlier incubation with cycloheximide above). GDP-mannose has been shown to be the donor of the mannose incorporated into endogenous acceptor(s) by particulate preparations from yeast [14–16].

These findings show that the effect of cyclo-

Table 4
Effect of cycloheximide on the incorporation of radioactivity from GDP-^{14}C-mannose into endogenous acceptor(s) by a particular preparation from *S. cerevisiae* LK2G12.

Cycloheximide (μg/ml)	GDP-mannose		Mannose incorporated (nmoles)	Relative activity
	(nmoles)	(nCi)		
0	0.33	50	0.030	100
4	0.33	50	0.029	95
96	0.33	50	0.028	93
0	80.0	30	1.120	100
96	80.0	30	1.230	110

Particulate preparation (130 μg protein) in 500 μl tris-maleate buffer pH 6.8–15 mM $MnCl_2$–1 mM mercaptoethanol was incubated with cycloheximide at 30° for 5 min. GDP-^{14}C-mannose was then added and the mixtures incubated at 30° for 30 min. The reaction was stopped by precipitation with 5% trichloroacetic acid at 0° for 30 min and the precipitate collected on glass filter discs and counted in a liquid scintillation spectrometer [5].

heximide is not on the synthesis or interconversion of sugars or their nucleotides, but somewhere in the steps leading to the synthesis or glycosylation of the acceptor molecules.

To determine if cycloheximide inhibits any of the enzyme reactions involved in the addition of mannose to the endogenous acceptor, we have employed a particulate preparation obtained by breakage of actively growing cells (results to be described elsewhere). Cycloheximide had no effect on the incorporation of the mannose moiety of GDP-mannose by the particulate preparation, even at a concentration 24 times that used to halt protein synthesis in the intact cell (table 4). It can also be seen that mannose incorporation was not affected by cycloheximide even when the concentration of GDP-mannose was increased 240-fold. The amount of mannose incorporated in this experiment is far in excess of the quantity of lipid or other intermediates that might be present. The lack of inhibition under these circumstances rules out the possibility that cycloheximide prevents in some way an intermediate from re-entering the reaction cycle. The trapping of the lipid intermediate in this manner has been shown by Siewert and Strominger [17] to be the primary event in the inhibition of peptido-glycan formation brought about by bacitracin.

4. Discussion

Our results have shown that both protein and mannan synthesis are highly sensitive to the antibiotic in cycloheximide-sensitive yeast and insensitive in the resistant species. In the presence of the drug, sensitive organisms accumulate GDP-mannose, the sugar donor for the mannan polymers, but glycosylation of the endogenous acceptor(s) by a particulate preparation is not affected. These findings, together with the known primary action of cycloheximide on protein synthesis [3, 11], are consistent with the following mechanism for inhibition of human synthesis. After addition of cycloheximide, formation of acceptor polypeptides is prevented, but glycosylation of those present is completed and these molecules are incorporated into the cell wall [6]. Glycosylation now stops for lack of acceptors and GDP-mannose accumulates since its synthesis is not inhibited.

These observations support the previous suggestion [6] that formation of the yeast wall is the result of two largely independent processes. Glucan is built independently of protein synthesis (at least initially), possibly in particles found on the outer plasmalemma surface [18, 19]. Mannan polypeptides account for a large part of the amorphous matrix of the wall. The data presented here support the hypothesis that the polypeptide moiety of mannan is synthesized on polyribosomes according to the established pathways of protein synthesis, with the carbohydrate chains probably added during transport of the macromolecules from the polysomes to the site of incorporation into the wall. Scherr and Uhr [20] and Caccam and Eylar [21] have proposed a similar pathway for the secretion of glycoproteins by mammalian cells.

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