

Echinocandin inhibition of 1,3- β -D-glucan synthase from *Candida albicans*

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The cyclic peptide antibiotic echinocandin was found to inhibit 1,3- β -D-glucan synthase activity present in a mixed membrane fraction from *Candida albicans*. Addition of antibiotic reduced the $V_{\max}$ of the enzyme, but the K_m was unaffected. GTP stimulated enzyme activity ~4-fold, but did not affect the percentage inhibition of the enzyme by echinocandin. Treatment of the reaction products with α -amylase and β -glucanase confirmed that the polymer synthesised was 1,3- β -D-glucan, not glycogen.

<i>Candida albicans</i>	Antibiotic	Echinocandin	1,3- β -D-Glucan synthase
	1,3- β -D-Glucanase	GTP	

1. INTRODUCTION

Echinocandins are a series of antibiotics produced by *Aspergillus nidulans* var. *echinulatus* [1]. Echinocandin B is highly active against yeasts, and against *Candida albicans* in particular, causing rapid lysis in growing cells [2]. The structure consists of a cyclic hexapeptide linked to a linoleic acid side chain [3]. A more stable and more active derivative, echinocandin 32528, has a stearic acid side chain, and an amino alkyl group linked to one of the amino acid residues [4].

Ultrastructural studies have shown that echinocandin B induces thinning of the newly formed yeast cell wall, together with the appearance of membranous bodies close to the plasma membrane of the emerging bud. Complete intracellular disorganisation follows, but no effect is observed on the mature cell wall [5].

Unlike the polyene antibiotics, echinocandin B does not cause leakage of K^+ from yeast cells [2]. Echinocandin 32528 inhibits incorporation of

radioactivity from glucose into trichloroacetic acid-insoluble material of *C. albicans*. The reduction in radiolabelling is associated with glucose polymer containing fractions of the cell [4].

1,3- β -D-Glucan is one of the major structural polymers in yeast cell walls, therefore inhibition of 1,3- β -D-glucan synthesis could result in lysis of growing cells. We show that 1,3- β -D-glucan synthase activity in a mixed membrane fraction from *C. albicans* is reduced by up to 80% in the presence of echinocandin 32528. The kinetics of the inhibition appear complex.

2. MATERIALS AND METHODS

Echinocandin 32528 was provided by Sandoz Forschungsinstitut, Vienna, Austria. UDP-[U- ^{14}C]glucose was obtained from Amersham International, England. GTP and α -amylase were purchased from Sigma. Zymolyase '60000' (a 1,3- β -D-glucanase preparation) was from Kirin Brewery, Takasaki, Japan.

2.1. Organism and culture conditions

C. albicans 6406 (obtained from the Mycological Reference Laboratory), an isolate from a human

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finger nail infection, was the organism used in all experiments. It was grown in Difco yeast nitrogen base medium (plus 2% glucose) at 37°C on an orbital shaker (190 rpm) to a cell density of 0.3 mg dry wt/ml.

2.2. Preparation of mixed membrane fraction

C. albicans (150 mg dry wt) was harvested, the cells washed with distilled water, then with 50 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 1.0 mM β -mercaptoethanol, 1.0 M sucrose, $\pm$ 25 μ M GTP (buffer A), and finally resuspended in 0.5 ml buffer A. Glass beads (0.45–0.5 mm) were added to just below the meniscus, and the cells were broken by agitation on a vortex mixer. Cell breakage was checked microscopically. Four periods of 1 min each were usually sufficient to break ~90% of the cells. Residual intact cells and cell wall debris were removed by centrifugation at $2700 \times g$ for 10 min. This pellet was washed with 3 ml buffer B (buffer A minus 1.0 M sucrose), and the glass beads were washed with 6 ml buffer B. The supernatant fluids were pooled and centrifuged at $100000 \times g$ for 45 min. The resulting pellet was washed by centrifugation in 10 ml buffer B, and resuspended at ~100 μ g protein per 10 μ l in buffer B (~0.5 ml), containing 33% glycerol. All operations were performed at 4°C [7].

The material, which consisted mainly of mixed membranes, was either used immediately as a source of enzyme, or stored at -80°C.

2.3. Assay procedure

The assay mixtures contained, in a total volume of 35 μ l, UDP-[U-¹⁴C]glucose (0.4–10.0 mM, 0.04–0.9 μ Ci/ μ mol), 100 μ M GTP, 0.8% BSA, 50 mM Tris-HCl (pH 8.0), and 10 μ l of the mixed membrane preparation containing ~100 μ g protein.

Echinocandin 32528 was added as required to the incubation mixtures. The reactions were incubated at 30°C, for up to 30 minutes, and then terminated by addition of 8 μ l glacial acetic acid. The reaction products were separated by descending paper chromatography on Whatman 3MM paper in 95% ethanol, 1.0 M acetic acid (7:3, v/v). Polymers formed during the incubation remained at the origin of the chromatogram. A 1 cm strip from this region of the chromatogram was air dried and immersed in PPO/toluene (4 g/l) and

assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrophotometer.

2.4. Enzymic breakdown of reaction products

In these experiments, the reaction was terminated by heating to 100°C. Fifty μ g of either α -amylase or zymolyase 60000 in 50 mM Tris-HCl (pH 7.5) were added to the incubation mixtures. After incubation for 18 h at room temperature, the reaction was terminated by addition of 0.1 ml of 10% trichloroacetic acid, and the radioactivity present in the chromatographically immobile material was determined.

3. RESULTS

3.1. Echinocandin inhibition of 1,3- β -D-glucan synthesis

Mixed membrane preparations containing 1,3- β -D-glucan synthase activity were incubated under the conditions described above, in the presence of echinocandin 32528. At a final antibiotic concentration of 10 μ g/ml and 100 μ g/ml in the assay, the activity of the enzyme was reduced by 25 and 80%, respectively (fig.1). Increasing the echinocandin

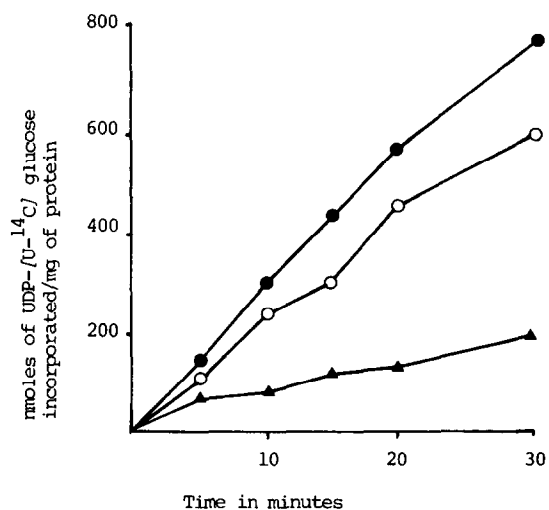


Fig.1. Effect of echinocandin on *C. albicans* 1,3- β -D-glucan synthase. The enzyme was prepared in the presence of 25 μ M GTP. The effects of echinocandin at concentrations of 10 μ g/ml (○—○) and 100 μ g/ml (▲—▲) on 1,3- β -D-glucan synthase in the presence of 100 μ M GTP, were compared with controls containing no antibiotic (●—●).

concentration above 100 $\mu\text{g/ml}$ did not result in a further reduction in enzyme activity.

Since UDP-glucose is also a precursor for glycogen and possibly other polymers in the yeast cell, it was necessary to determine whether the polymer produced was indeed a 1,3- β -D-glucan.

The reaction products were resistant to digestion by α -amylase, but 75% of the radioactivity associated with chromatographically immobile material was released by incubation with zymolyase 60000 (fig.2). Zymolyase 60000 is a 1,3- β -D-glucanase containing traces of protease and mannanase, but no α -amylase activity [4]. Analysis by Smith degradation of the reaction products from a similar assay confirmed that the polymer produced was a 1,3- β -D-glucan [6].

3.2. Kinetics of inhibition

The kinetic parameters of 1,3- β -D-glucan synthase inhibition by echinocandin were assessed by direct linear plot, and by Lineweaver-Burk plot (fig.3). The addition of echinocandin 32528 to the assay mixture did not affect the apparent K_m of the enzyme, but reduced the $V_{\max}$ (table 1). This situa-

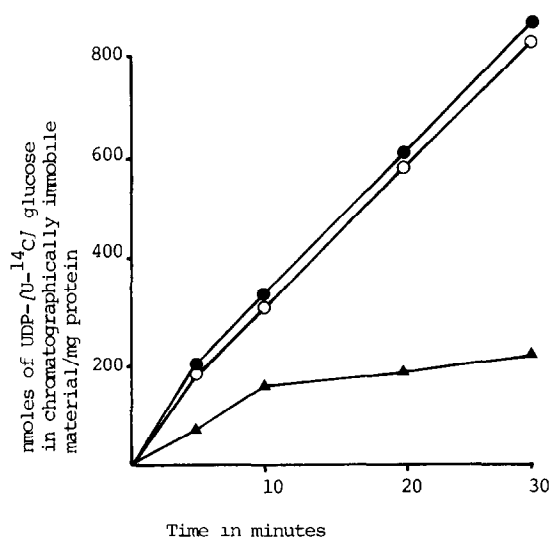


Fig.2. Effect of lytic enzymes on the release of radioactivity from products of the 1,3- β -D-glucan synthase reaction. The enzyme was prepared in the presence of 25 μM GTP, and GTP (100 μM) was included in the reaction mixtures. Control (●—●), α -amylase-treated (○—○) and zymolyase 60000-treated (▲—▲) preparations were analysed as described in section 2.4.

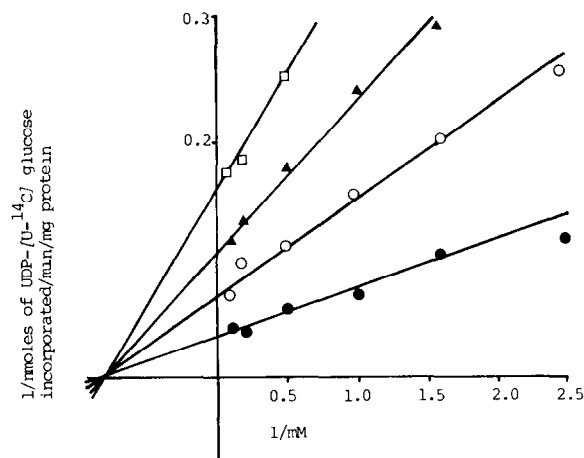


Fig.3. Lineweaver-Burk plot of echinocandin inhibition of *C. albicans* 1,3- β -D-glucan synthase. The enzyme was prepared in the presence of 25 μM GTP. Echinocandin was added to assay mixtures (containing 100 μM GTP) to final concentrations of 10 $\mu\text{g/ml}$ (○—○), 50 $\mu\text{g/ml}$ (▲—▲) and 200 $\mu\text{g/ml}$ (□—□). These and the control reactions (●—●) were incubated at 30°C for 5 min. The reaction products were analysed as described in section 2.3.

tion is characteristic of non-competitive inhibition.

However, the inhibitor constant (K_i) was variable, and a plot of $1/v$ against antibiotic concentration yielded a curve, indicating that the kinetics of this system are not simply non-competitive (not shown). There are difficulties in interpreting the kinetic data, since the enzyme is present in a mixed membrane fraction, and conclusive evidence of the mechanism by which echinocandin 32528 inhibits 1,3- β -D-glucan synthase activity awaits the purification of the enzyme from *C. albicans*. So far, all attempts to do this have failed (P.A.B. Orlean, personal communication).

3.3. The effect of GTP on 1,3- β -D-glucan synthase activity

Preparation of the enzyme fraction from *C. albicans* in buffers containing GTP resulted in a 3-fold increase in the activity of the enzyme [4,7]. Inclusion of 100 μM GTP in assay mixtures containing enzyme prepared in the absence of GTP caused an increase in the specific activity of the enzyme from 3 to 8 nmol/min per mg protein (not shown). However, the percentage inhibition of

Table 1

GTP effect on 1,3- β -D-glucan synthase from *C. albicans*

Enzyme preparation	Initial specific activity ^a	Echinocandin (μ g/ml)	% Inhibition	K_m (mM)	V_{max} ^b
Standard	8.1	0		1.2	22
		10	20	1.2	19
		100	75	1.2	12
GTP	35.0	0		1.2	27
		10	25	1.2	14
		100	80	1.2	6

^a nmol UDP-[U-¹⁴C]glucose incorporated/min per mg protein^b 1/nmol UDP-[U-¹⁴C]glucose incorporated/min per mg proteinAll assay mixtures contained 100 μ M GTP

1,3- β -D-glucan synthase by echinocandin was similar in the presence or absence of GTP (table 1). Although echinocandin had a greater effect on the V_{max} of the enzyme fraction prepared in the presence of GTP, the pattern of enzyme inhibition was not affected.

4. DISCUSSION

On incubation with GTP, the specific activity of *C. albicans* 1,3- β -D-glucan synthase was found to be 35 nmol/min per mg protein. This is comparable with the specific activity of 1,3- β -D-glucan synthase from a glycogen synthase-less mutant of *Saccharomyces cerevisiae* [8]. Authors in [8] calculated that this figure represented a significant proportion (20%) of the total 1,3- β -D-glucan synthetic capacity of the *S. cerevisiae* cell. The only available analysis of *C. albicans* cell walls suggests that 1,6- β -D-glucan, and not 1,3- β -D-glucan, is the major glucose polymer present [9]. Therefore a large percentage of in vivo *C. albicans* 1,3- β -D-glucan synthase activity is apparently retained here in vitro.

At an echinocandin concentration of ~ 50 μ g/ml in the assay, there was 50% inhibition of the enzyme. The minimum inhibitory concentration of echinocandin 32528 for *C. albicans* 6406 in batch culture is 0.05 μ g/ml (cell dry wt, 27 μ g/ml). At in-

creased cell densities, a greater concentration of antibiotic was required to prevent growth of the organism (more than 1:1), although the exact relationship between inoculum density and echinocandin concentration was unclear [4]. Considering cell densities used in preparation of the membrane fraction, the antibiotic appeared to be more active in vitro than in vivo. The difference between the concentration of antibiotic required to inhibit cell growth in vivo, and inhibit glucosyltransferase in vitro, may reflect changes in the accessibility of the active site of the enzyme, which occurs on cell breakage and fractionation.

Previous work has shown that echinocandin does not affect the incorporation of chitin and mannan precursors into their respective polymers in *C. albicans* [2]. Other possible targets of echinocandin action include the plasma membrane and endogenous β -glucanases in the cell wall. As already mentioned, echinocandin does not cause leakage of K⁺ from *C. albicans* [2]. Amino acid and peptide transport are also unaffected [4], suggesting that overall membrane damage does not occur. Ultrastructural evidence shows that increased secretion of lytic enzymes might take place, resulting in cell lysis. However, activation of β -glucanases results in increased incorporation of radioactivity from glucose into trichloroacetic acid-insoluble cellular material, rather than the opposite [4,10].

Papulacandin B and aculeacin A appear to have a similar mode of action to echinocandin. Both of these antibiotics inhibit 1,3- β -D-glucan synthase activity from *Geotrichum lactis*, but do not cause K⁺ leakage or changes in β -glucanase activities [11].

The kinetic data available at present are insufficient to explain the mechanisms of echinocandin inhibition of *C. albicans* 1,3- β -D-glucan synthase. As the enzyme is present in a mixed membrane fraction, it is not known whether any membrane associated activities affect the interaction of echinocandin with the enzyme.

Authors in [12] suggested that papulacandin B may be modified in the cell prior to interaction with the enzyme, and that this is one possible reason for lack of papulacandin inhibition of glucosyltransferase from *S. cerevisiae*. However, it is possible that any papulacandin-sensitive steps in 1,3- β -D-glucan synthesis were not preserved in

their experimental system [10]. A small variation in the procedure for obtaining a membrane fraction from *C. albicans* (e.g., omission of β -mercaptoethanol or sucrose from the preparation buffers) can result in an active 1,3- β -D-glucan synthase preparation insensitive to echinocandin [4]. The reason for this apparent difference in echinocandin sensitivity is not known and requires further investigation.

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