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W-1 Solubilization and Kinetics of Inhibition by Cilofungin of *Candida albicans* (1,3)-β-D-Glucan Synthase

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(1,3)-β-D-Glucan synthase of *Candida albicans* was rendered soluble by treatment of membrane preparations with the polyoxyethylene ether detergent W-1. Extraction with 0.025% W-1 at 4°C for 24 h effectively solubilized and activated the enzyme. Under these conditions, >85% of the protein in membrane preparations was released, and about 64% of the glucan synthase activity could be recovered in the soluble form. Soluble enzyme activity was stable for more than 12 days at 4°C. Also, glucan synthase activity in the extracted membrane preparations could be activated to achieve more than twice the enzyme activity in the original, unextracted membrane preparations. The soluble glucan synthase had characteristics similar to those of the membrane-bound enzyme. Soluble glucan synthase had an apparent K_m of 2.0 mM, and particulate glucan synthase had an apparent K_m of 2.5 mM. Kinetics of cilofungin inhibition for both enzyme preparations were noncompetitive, with an apparent K_i of 2.5 μM; both preparations could be inhibited by cilofungin but not by its peptide nucleus or side chain, either alone or in combination. The reaction products from both forms of the enzyme were sensitive to (1,3)-β-D-glucanase degradation but not to α-amylase, α-glucosidase, or proteinase K degradation and thus were shown to be β(1→3) glucan.

Cilofungin (LY121019) is a lipopeptide antifungal agent, the specific activity of which against *Candida albicans*, *Candida tropicalis*, and *Neurospora crassa* is well documented (4–6, 8, 17, 25). Its target, (1,3)-β-D-glucan synthase (EC 2.4.1.34; UDPglucose:1,3-β-D-glucan 3-β-D-glucosyltransferase), is a membrane-bound enzyme that catalyzes the synthesis of β(1→3) glucan, one of the major cell wall components of *Candida* spp. Studies of cilofungin and its target glucan synthase in *C. albicans* are few. Taft et al. (27) demonstrated the mode of action of cilofungin against glucan synthase in *C. albicans*, while Sawistowska-Schröder et al. (21) used echinocandin to inhibit *C. albicans* glucan synthase. Both studies were done with cell enzyme extracts. Sawistowska-Schröder indicated that kinetic studies were difficult, perhaps because of the particulate nature of the enzyme preparations.

While a number of detergents have been used to solubilize glucan synthase from fungi, yeasts, and plants (2, 3, 7, 9, 10, 18, 20), up to the present time no successful solubilization studies of glucan synthase from *C. albicans* have been reported. In this study, we explored the *C. albicans* glucan synthase solubilization, its properties, and the kinetics of its inhibition by cilofungin and echinocandin B.

MATERIALS AND METHODS

Organisms and media. *C. albicans* A26 was a clinical isolate. This strain was used for both in vitro and in vivo studies by Gordee et al. (4). Stock cultures of *C. albicans* were kept frozen in lactose-glycerol medium at −80°C. Sabouraud dextrose broth (Difco, Detroit, Mich.) or agar was used as the culture medium.

Chemicals and reagents. UDP-[U-^{14}C]glucose (228 mCi/mmol) was purchased from ICN Pharmaceuticals (Irvine, Calif.). Proteinase K and pronase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Novozym 234 was purchased from Calbiochem (La Jolla, Calif.). Cilofungin, echinocandin B, and echinocandin B nucleus and side chain were supplied by Eli Lilly & Co. (Indianapolis, Ind.). All other chemicals, enzymes, and detergents were purchased from Sigma (St. Louis, Mo.).

Protoplast preparations. Thirty milliliters of *C. albicans* overnight cultures was inoculated into 2 liters of prewarmed Sabouraud dextrose broth and incubated at 37°C with shaking for 5 h. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C.

Protoplasts were prepared by the methods of Quigley et al. (19) and Taft et al. (27). In brief, harvested cells were resuspended in the pretreatment medium of Miragal et al. (15) (5 mM EDTA [pH 9], 50 mM dithiothreitol, 0.5 mg of pronase per ml) for 30 min at 28°C with shaking. Cells were harvested and washed once with cold 0.6 M KCl in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.0). The pretreated cells were subjected to Novozym 234 digestion (10 mg/ml in 0.6 M KCl) at 28°C with shaking. Greater than 95% of the protoplasts were released following 20 to 30 min of Novozym incubation (as judged by microscopic examination and hypotonic lysis).

Protoplasts were harvested by centrifugation (1,500 × *g* for 10 min at 4°C) and washed twice with cold 1.2 M sorbitol in 20 mM HEPES buffer (pH 7.0). Aliquots of protoplast pellets were rapidly frozen in dry ice-methanol and stored at −80°C until use. Protoplasts prepared in this way were stable for more than 2 months.

Enzyme preparations. Frozen protoplasts were thawed and broken by vortexing with 0.3-mm glass beads in buffer A (50 mM Tris hydrochloride [pH 8.0], 1 mM dithiothreitol, 5 mM EDTA, 10 mM sodium fluoride [NaF], 100 μM GTP with 1 M sucrose. The resultant preparation was defined as the crude lysate. In some experiments, crude lysates were

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centrifuged at 100,000 × *g* at 4°C for 1 h. The pellets were washed once with buffer A with 1 M sucrose and resuspended in the same solution; this preparation was defined as the membrane preparation.

Detergent solubilization of enzyme. In the initial solubilization experiments, crude lysates (1 mg of protein per ml) were treated with BIG CHAP (*N*,*N*-bis[3-D-glucon-amidopropyl] cholamide), Triton X-100 (TX-100), or the polyoxyethylene ether (W-1) at 0.01 to 0.1% in the presence of 0.1% bovine serum albumin (BSA) at 4°C for 24 h. The extracted crude lysates were centrifuged at 100,000 × *g* at 4°C for 1 h. The resulting supernatants and pellets, along with the unextracted crude lysates and the extracted crude lysates, were assayed for glucan synthase activity.

In later experiments, W-1 at 0.025% was used to extract the washed 100,000 × *g* membrane preparations (2.5 mg of protein per ml) in the absence of BSA. Enzyme activity at extraction times of 24 to 144 h was monitored. Soluble glucan synthase in the 24-h extracted supernatants was kept at 4°C for up to 288 h to examine enzyme lability.

The kinetics of soluble glucan synthase activity with or without inhibitors and reaction products were compared with those of particulate glucan synthase activity.

Glucan synthase activity assay. Glucan synthase activity was assayed by the methods of Taft et al. (27) with some modifications. For each assay, 25 μl of reaction mixtures containing 50 μg of α-amylase, various UDP-glucose concentrations (0.125 to 2 mM), and UDP-[^{14}C]glucose (30,000 to 100,000 dpm) was added to 75 μl of enzyme preparations (6 to 130 μg of protein). In some experiments, 0.1% BSA was used in the assay. The protein concentration was determined by the method of Bradford (1).

In glucan synthase kinetics studies, enzyme preparations were incubated with reaction mixtures at room temperature. Reaction rates were measured for 0 to 15 min. The reactions were stopped by the addition of 1 ml of ice-cold 10% trichloroacetic acid. Reaction products were placed on membrane filters (0.45-μm-pore size; type HA; Millipore Corp., Bedford, Mass.). Filters were washed with cold 10% trichloroacetic acid and dried, scintillation fluid (Ready Protein; Beckman, Fullerton, Calif.) was added, and the samples were counted.

In glucan synthase inhibition assays, cilofungin and other potential inhibitors at 10 μg/ml were preincubated with enzyme preparations at room temperature for 5 min before reaction mixtures were added. In control groups, enzyme preparations were preincubated with solvent, i.e., methyl sulfoxide or dimethyl sulfoxide, at the same concentrations as those used in the inhibitor solutions.

Characterization of reaction products. Reaction products of glucan synthase were evaluated by specific enzyme degradation experiments. Glucan synthase reactions were stopped after 12 min of incubation by 5 min of boiling. The reaction products were recovered by centrifugation at 12,000 × *g* at 4°C for 15 min. The pellets were washed once with distilled water and treated with (i) 100 μl of 50 mM sodium acetate buffer (pH 5.4) containing 100 μg of 1,3-β-D-exoglucanase (mollusk) prepared by the methods of Hrmova et al. (9), (ii) 100 μl of 50 mM Tris hydrochloride buffer (pH 7.5) containing 100 μg of α-amylase, α-glucosidase, or proteinase K, or (iii) 100 μl of sodium acetate buffer or Tris hydrochloride buffer only. Enzyme degradations were carried out at 37°C for 16 h, and results were compared with those of undigested controls. Reactions were terminated by the addition of 1 ml of glacial acetic acid (1:1 with water). Pellets recovered by centrifugation were resuspended in 1 ml of cold 10% trichloroacetic acid and placed on membrane filters as described above.

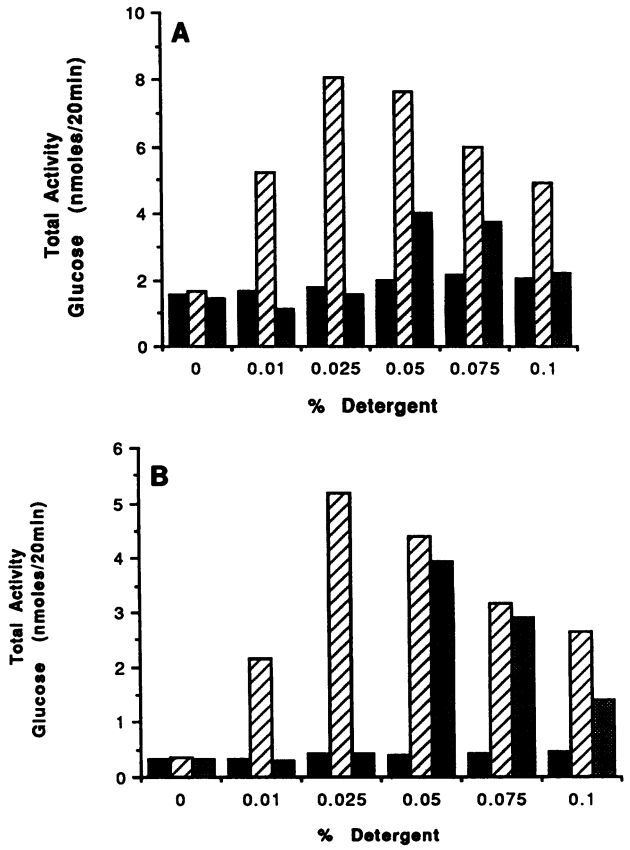

FIG. 1. (A) Activation of glucan synthase in crude lysates by BIG CHAP (■), W-1 (▨), or TX-100 (▩). Crude lysates (130 μg of protein) were incubated with various concentrations of BIG CHAP, W-1, or TX-100 at 4°C for 24 h. Glucan synthase activity was determined with 2 mM UDP-glucose as the substrate (92,000 dpm per assay). Extractions and assays were done in the presence of 0.1% BSA. (B) Release of soluble glucan synthase by BIG CHAP (■), W-1 (▨), or TX-100 (▩). Crude lysates extracted with BIG CHAP, W-1, or TX-100 at 4°C for 24 h were centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting supernatants were assayed for soluble glucan synthase activity with 2 mM UDP-glucose as the substrate (92,000 dpm per assay). Extractions and assays were done in the presence of 0.1% BSA.

RESULTS

Detergent activation and solubilization of glucan synthase in crude lysates. The activation and solubilization of glucan synthase activity in crude lysates by BIG CHAP, TX-100, or W-1 were compared by use of a 24-h extraction time in the presence of 0.1% BSA (Fig. 1A and B). BIG CHAP had no activation or solubilization effects on glucan synthase activity.

TX-100 at 0.05% activated enzyme activity by twofold, as compared with the enzyme activity in the 24-h unextracted crude lysate preparations. It is worth noting that the glucan synthase activity in the 24-h crude lysate preparations was only half that in the fresh crude lysate preparations (Table 1). The soluble glucan synthase activity in the extracted 100,000 × *g* supernatants was approximately 10-fold higher than that in the unextracted supernatants.

TABLE 1. Characterization of glucan synthase from different fractions[a]

Fraction (μg)	Total activity (nmol/min)	% Glucan synthase activity	$K_{m\ app}$ (mM)	V_{max} (nmol/min/mg)	Half-life (h)
Crude (104.6)	0.139	100	0.25	1.7	24
Membrane (control) (57.2)	0.161	116	0.24	3.3	>144
Membranes extracted with W-1 (54.8)	0.354	255	0.20	8.0	>192
100,000 × *g* supernatants from membranes extracted with W-1 (48.2)	0.143	103	0.20	3.7	>288
100,000 × *g* pellets from membranes extracted with W-1 (6.3)	0.080	58	0.19	15.2	>288

[a] Solubilization was done with membrane preparations and 0.025% W-1 at 4°C for 24 h. The assay was done in the presence of 0.1% BSA and various UDP-glucose concentrations (0.125 to 2 mM) (42,000 dpm per assay). Total activity was calculated with 1 mM UDP-glucose. Glucan synthase activity was a percentage of that in crude lysates (total activity).

W-1 at 0.025% activated enzyme activity by fourfold, as compared with the enzyme activity in the 24-h unextracted crude lysate preparations. The soluble glucan synthase activity in the extracted supernatants was approximately 12-fold higher than that in the unextracted supernatants.

Extraction times shorter than 24 h, i.e., 2, 4, or 6 h, resulted in less soluble glucan synthase activity and less protein released (data not shown).

W-1 solubilization of glucan synthase in membrane preparations. The effect of different W-1 extraction times on glucan synthase activity in the membrane preparations was also tested. W-1 was used at a final concentration of 0.025%, and the extractions were done at 4°C for 24 to 144 h. Glucan synthase activity in the extracted membrane preparations increased over time, and the highest enzyme activity was seen with 120 h of extraction (Fig. 2). Enzyme activity in the 120-h extracted membrane preparations was 1.5-fold higher than that in the 24-h extracted membrane preparations. Soluble glucan synthase activity following 24 h of W-1 extraction also increased over time and reached its highest level at 120 h. Glucan synthase activity in the unextracted membrane preparations decreased and reached its half-life at approximately 144 h.

With a 24-hour extraction time, >85% of the protein in the membrane preparations was released into the supernatants. Also, approximately 64% of the glucan synthase activity was recovered in the soluble form. This value was calculated as follows: total enzyme activity in the supernatants/(enzyme activity in the supernatants + enzyme activity in the pellets) (calculated with data in Table 1).

Characterization of soluble glucan synthase and comparison with the particulate form. The kinetics and the half-lives of glucan synthase from crude lysates, unextracted membranes (controls), membranes extracted with W-1, 100,000 × *g* supernatants from membranes extracted with W-1, and 100,000 × *g* pellets from membranes extracted with W-1 were compared (Table 1). The apparent K_m ($K_{m\ app}$) values of glucan synthase in different fractions were all similar, 0.2 to 0.25 mM (Fig. 3). Using the apparent V_{max} ($V_{max\ app}$) values of the fresh crude lysates (1.7 nmol/min/mg of protein) as our standards, we found the glucan synthase activity in the membrane preparations to be almost twofold higher with a $V_{max\ app}$ of 3.3 nmol/min/mg of protein. A further increase in the enzyme activity was seen when membrane preparations were extracted with W-1. Membrane preparations extracted with W-1 had a $V_{max\ app}$ of 8 nmol/min/mg of

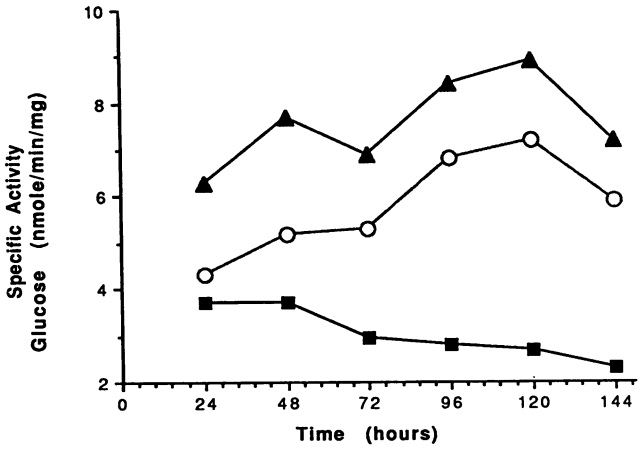

FIG. 2. Glucan synthase lability. Unextracted membranes (controls) (■) or membrane preparations extracted with W-1 (▲) and the 100,000 × *g* supernatants recovered from membrane preparations extracted with W-1 (○) for 24 h were kept at 4°C. Aliquots were taken and assayed for glucan synthase activity for 6 days with 1 mM UDP-glucose as the substrate (40,000 to 50,000 dpm per assay) in the presence of 0.1% BSA.

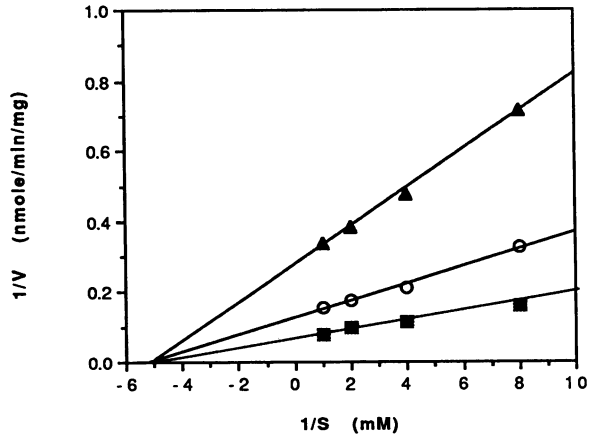

FIG. 3. Lineweaver-Burk plot of glucan synthase from membranes extracted with W-1 (○), supernatants from membranes extracted with W-1 (▲), and pellets from membranes extracted with W-1 (■). Membrane preparations extracted with 0.025% W-1 at 4°C for 24 h were centrifuged at 100,000 × *g* for 1 h at 4°C. Glucan synthase activity in extracted membrane preparations, 100,000 × *g* supernatants, and 100,000 × *g* pellets was assayed with UDP-glucose as the substrate (0.25 to 2 mM) (42,000 dpm per assay) in the presence of 0.1% BSA.

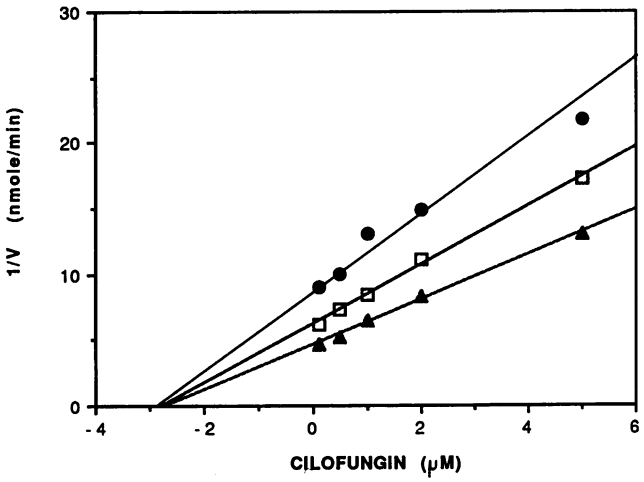

FIG. 4. Dixon plot of cilofungin K_i. Crude lysates were incubated with cilofungin at 0.1, 0.5, 1, 2, or 5 μg/ml at room temperature for 5 min. Reaction mixtures containing UDP-glucose at 0.125 mM (●), 0.25 mM (□), or 0.5 mM (▲) (49,000 dpm per assay) were added. Reaction rates were measured for 0 to 15 min.

protein, a value 4.5-fold higher than that for the crude lysates. Soluble glucan synthase had a $V_{max\ app}$ of 3.7 nmol/min/mg protein. This value was twofold higher than that for the crude lysates. The residual glucan synthase in the 100,000 × *g* pellets had a $V_{max\ app}$ of 15.2 nmol/min/mg of protein, a value almost ninefold higher than that for the crude lysates.

The half-lives of glucan synthase in different fractions from membrane solubilization studies are shown in Table 1. The half-lives of the enzyme were approximately 24 h in crude lysates and approximately 144 h in unextracted membrane preparations. The half-lives of glucan synthase were >192 h in membrane preparations extracted with W-1 and >288 h in both 100,000 × *g* supernatants and 100,000 × *g* pellets from membrane preparations extracted with W-1.

Dixon plots of cilofungin inhibition of glucan synthase in crude lysates revealed that cilofungin acts as a noncompetitive inhibitor, with an apparent K_i ($K_{i\ app}$) of 2.5 ± 0.3 μM (Fig. 4). The soluble glucan synthase $K_{i\ app}$ values were identical to the $K_{i\ app}$ values of the particulate glucan synthase (data not shown). Like the particulate glucan synthase, the soluble glucan synthase was inhibited by cilofungin; e.g., at 10 μg/ml, >70% enzyme inhibition was seen, but neither form of the enzyme was inhibited by the cilofungin components (i.e., the echinocandin B nucleus or the lipophilic side chain, either alone or in combination) at the same concentrations (data not shown). Echinocandin B was also shown to be a noncompetitive inhibitor of glucan synthase in crude lysates, with a $K_{i\ app}$ of 3.3 ± 0.9 μM.

The reaction products of both forms of glucan synthase were shown not to be glycogen, α-glucan, or protein through their inability to be degraded by α-amylase, α-glucosidase, or proteinase K. The reaction products were degraded >90% by (1,3)-β-D-glucanase and thus appeared to be β(1→3) glucan (data not shown).

DISCUSSION

Detergent solubilization conditions were developed to release membrane-bound glucan synthase from *C. albicans*. The use of W-1 at 0.025% could effectively solubilize glucan synthase from the membrane preparations when extraction was carried out at 4°C.

It is interesting to note that the half-life of the soluble glucan synthase was considerably longer than the half-life of the glucan synthase in the crude lysates (>288 h versus 24 h). Several factors might have contributed to this observation. First, it is accepted that glucan synthase is a membrane-bound enzyme (11, 13, 14, 16, 22), and the use of washed membrane preparations may prolong the enzyme half-life by removing proteases present in the crude lysates. Second, it is well documented that GTP, EDTA, and NaF activate glucan synthase activity and that sucrose stabilizes the enzyme activity (3, 12, 16, 22–24). The use of buffer A, which contains GTP, EDTA, and NaF, with 1 M sucrose throughout the extraction process may help maintain the enzyme in its active form. Finally, unknown mechanisms of activation of the glucan synthase by W-1 may also help prolong the enzyme half-life.

The inability of the molecular components of cilofungin to inhibit the membrane-bound glucan synthase was expected (26). We had hypothesized that the lipophilic side chain may help position the intact cilofungin in the cytoplasmic membrane for the peptide nucleus to exert its inhibitory action on the proximal glucan synthase. We also hypothesized that the peptide nucleus alone would inhibit the soluble glucan synthase. Our data suggest that the lipophilic side chain is required for the positioning of cilofungin on glucan synthase to bring about enzyme inhibition.

$K_{m\ app}$ values of *C. albicans* glucan synthase were reported to be 1.2 to 1.9 mM by Orleans (16) and Sawistowska-Schröder et al. (21). These values were somewhat higher than the $K_{m\ app}$ values that we reported here. The differences might be partly due to the different strains of *C. albicans* and the different methodologies used in preparing enzyme extracts. A $K_{i\ app}$ value of 2.5 μM for *C. albicans* glucan synthase, in either soluble or particulate form, for cilofungin is in agreement with that previously reported for membrane-bound preparations (27).

In conclusion, the soluble enzyme released by W-1 treatment of *C. albicans* membrane preparations was demonstrated to possess properties nearly identical to those of the particulate *C. albicans* glucan synthase. Both had similar enzyme kinetics and inhibition kinetics, and the reaction products from both forms of the glucan synthase appeared to be β(1→3) glucan. We hope that the solubilization of the active glucan synthase will allow us to proceed with more complete enzyme analyses and will aid in the search for more effective antifungal agents.

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