Functional reconstitution of a purified proline permease from *Candida albicans*: interaction with the antifungal cispentacin

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We have purified proline permease to homogeneity from *Candida albicans* using an L-proline-linked agarose matrix as an affinity column. The eluted protein produced two bands of 64 and 67 kDa by SDS-PAGE, whereas it produced a single band of 67 kDa by native PAGE and Western blotting. The apparent K_m for L-proline binding to the purified protein was 153 μ M. The purified permease was reconstituted into proteoliposomes and its functionality was tested by imposing a valinomycin-induced membrane potential. The main features of L-proline transport in reconstituted systems, viz. specificity and sensitivity to *N*-ethylmaleimide, were very similar to those of intact cells. The antifungal cispentacin, which enters *C. albicans* cells via an inducible proline permease, competitively inhibited the L-proline binding and translocation in reconstituted proteoliposomes. However, the uptake of L-proline in proteoliposomes reconstituted with the purified protein displayed monophasic kinetics with an apparent K_m of 40 μ M.

Keywords: L-proline, amino acid transport, proteoliposomes, cispentacin, *Candida albicans*

INTRODUCTION

Among several factors which affect yeast to mycelial transition of the pathogenic pleiomorphic yeast *Candida albicans*, various nutrients (sugars, amino acids, and other nitrogen sources, etc.) play an important role (Odds, 1988; Shepherd, 1991). Amino acids which enter metabolism by their conversion to 2-oxoglutarate are generally more active in inducing germ tube formation in *C. albicans* (Shepherd, 1991). L-Proline, one such amino acid, is known to be an efficient germ tube stimulator. Hence, earlier studies encompassing the characterization of transport of L-proline have regained importance (Jayakumar *et al.*, 1978, 1979, 1981; Dabrowa & Howard, 1981; Holmes & Shepherd, 1987). It has been established that the electrochemical gradient of protons generated by the plasma membrane (PM) ATPase of *C. albicans* is the main

driving force for the uptake and intracellular accumulation of L-proline as well as of other amino acids (Prasad, 1987).

The kinetics of proline transport in *Saccharomyces cerevisiae* have revealed that the uptake of proline occurs through two pathways; via a general amino acid permease which mediates the uptake of a wide variety of D and L amino acids including L-proline (Lasko & Brandriss, 1981), and via a specific permease possessing a high affinity for proline (Grenson *et al.*, 1970). In *C. albicans*, the transport of proline is mediated by an inducible system, which is rather unique, since no other amino acid transporter in yeast has so far been shown to elicit inducibility (Jayakumar *et al.*, 1978, 1979, 1981; Dabrowa & Howard, 1981; Holmes & Shepherd, 1987).

Notwithstanding the importance of membrane transport in the overall physiology of *C. albicans* and in drug design, very limited knowledge of its transporters exists (Prasad, 1987; Cooper, 1982; Payne & Shallow, 1985; Naider & Becker, 1990). Although none of the permease or membrane proteins mediating amino acid transport have been isolated and purified from yeast, a few permease-specific genes have been cloned and sequenced

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Abbreviations: AZA, azetidine-2-carboxylic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CM, crude membrane; DHP, 2,4-dehydro-DL-proline; NEM, *N*-ethylmaleimide; PM, plasma membrane.

from *S. cerevisiae* (Schmidt *et al.*, 1994 and references therein). The gene responsible for basic amino acid transport in *C. albicans* has also recently been cloned by functional complementation of the *can1* mutant of *S. cerevisiae* (Sychrova & Chevaller, 1993). *CAN1* of *C. albicans* has an ORF of 1713 nucleotides, encoding a protein of 571 amino acids with a calculated molecular mass of 63.3 kDa (Sychrova & Souciet, 1994). Generally, the size of yeast amino acid transport permeases deduced from their primary structures ranges between 64 and 68 kDa. The amino acid sequence of the permeases displays a high degree of hydrophobicity with 10–12 membrane-spanning stretches (Schmidt *et al.*, 1994).

To understand the functional processes of specific amino acid transporters, it is, however, essential that the protein in question is isolated and purified in its active form from the native membranes. The lack of an easy binding assay and the low amount of these transport proteins in the PM, coupled with their extreme hydrophobicity, have so far severely hampered attempts to purify them. For the first time we have succeeded in purifying an L-proline permease from *C. albicans* to homogeneity in a single step by using affinity chromatography. The assessment of the characteristics of L-proline binding to the purified protein and of its uptake kinetics into reconstituted proteoliposomes is the subject of this paper.

METHODS

Strain, growth conditions and materials. *Candida albicans* strain ATCC 10261 was grown and cultured in YEPD medium (2% peptone, 1% yeast extract and 2% glucose) (Rao *et al.*, 1986; Prasad & Höfer, 1987).

L-Proline agarose and Tween 40 were from Sigma. L-[¹⁴C]Proline (specific activity 9.250 GBq mmol⁻¹) was from Bhabha Atomic Research Center. All other reagents and chemicals of the highest purity grade were procured from local resources.

Transport of L-proline in the intact cells. Transport of L-proline was assayed as described in our earlier publications (Jayakumar *et al.*, 1978, 1979, 1981).

Isolation of the crude membrane (CM). Cells grown to mid-exponential phase in YEPD medium (1 g wet wt) were suspended in 2 ml grinding medium (250 mM sucrose, 10 mM Tris/HCl, pH 7.5, 1 mM PMSF) and 2 g glass beads (0.45–0.50 mm diameter). The suspension was mechanically disrupted in an MSK Braun cell homogenizer by agitating it for a total of nine cycles of 5 s each at 4000 vibrations min⁻¹. The homogenate was collected and centrifuged at 1000 g for 5 min at 4 °C to remove unbroken cells and glass beads. The supernatant was then centrifuged for 40 min at 15000 g at 4 °C and the resulting CM pellet was resuspended in 10 mM Tris/HCl (pH 7.5). The CM fraction was stored at -70 °C until further use.

Isolation of the purified PM fraction. The CM fraction was diluted with Percoll (18%, v/v) in 10 mM Tris/HCl (pH 7.5). The suspension was centrifuged at 40000 g for 40 min at 4 °C in a Sorvall TH-641 rotor (Hubbard *et al.*, 1986). The gradient so obtained had a translucent lipid layer on the top and a PM band just below it. The PM was aspirated and washed twice

with 10 mM Tris/HCl (pH 7.5) by centrifugation at 100000 g for 30 min in the same rotor to remove traces of Percoll. The purified PM pellet was resuspended in 10 mM Tris/HCl (pH 7.5) and the purity of the PM was routinely checked by assaying H⁺-ATPase at pH 6.6 and 9.0 (optimum pH for mitochondrial ATPase). The ratio of the two activities ($P = R_{6.6}/R_{9.0}$) was always high (> 30), implying almost no contamination by the mitochondrial fraction (Gupta *et al.*, 1991).

Solubilization and purification of L-proline permease. The purified PM in a total volume of 3 ml (3–4 mg protein ml⁻¹) was solubilized using Tween 40 (0.7%, v/v) as a detergent in the solubilization buffer containing 10 mM Tris/HCl (pH 7.5), 1 mM PMSF, 1 mM EDTA and 2 mM 2-mercaptoethanol. The samples were incubated at 30 °C for 5 min and then sonicated for 3–4 min in a bath sonicator (Julabo, Seelbach) at 30 °C. The solubilized proteins (7–10 ml) were separated from the unsolubilized protein by centrifugation at 40000 g for 30 min in a Sorvall centrifuge using a TH-641 rotor. The soluble protein present in the supernatant was dialysed for 12 h against 10 mM Tris/HCl (pH 7.5) to remove additives like PMSF, EDTA and 2-mercaptoethanol. The dialysis also resulted in the partial removal of the detergent; however, the detergent concentration remained above its critical micellar concentration value and the solubilized protein stayed in solution. The dialysed protein fraction (7–8 mg) was loaded onto an L-proline-linked agarose column (5 ml) equilibrated with 100 mM Tris/citrate (pH 5.5) containing 0.1% Tween 40. The unbound protein was eluted with 10 bed volumes (50 ml) of this buffer. The bound protein was then eluted with 3–4 bed volumes (20–25 ml) 2 M NaCl (containing 0.1% Tween 40). Each eluted fraction of 5 ml was dialysed for 12 h against 10 mM Tris/HCl (pH 7.5) to remove NaCl before the binding assay.

Equilibrium dialysis for the binding assay. In order to monitor the purification of proline permease, it was essential to develop a reliable assay. Using the Dianorm equilibrium dialysis system, it was possible to do several binding assays simultaneously. L-Proline binding to CM, PM and purified protein was done routinely using equilibrium dialysis. The two teflon chambers of each dialyser unit were separated by dialysis membrane with a 1000 Da cut-off (no. 10.16; Dianorm) which was earlier soaked in 10 mM Tris/citrate (pH 5.5) for 5 min. One of the chambers was filled with membrane protein (200 µg CM or PM or 1–2 µg purified protein), 100 mM Tris/citrate (pH 5.5) and 10 µM (unless otherwise mentioned) L-[¹⁴C]proline (925 MBq mmol⁻¹) in a total volume of 1 ml; the other chamber contained only buffer (100 mM Tris/HCl, pH 5.5). The dialyser unit was submerged in a 30 °C water-bath and rotated at 12 r.p.m. for 12–14 h. The solutions were collected from each chamber and the radioactivity of a 10 µl aliquot was counted by using a Beckman beta liquid scintillation counter with a detergent-based scintillation cocktail. The binding was calculated from the difference in c.p.m. obtained between the two chambers after equilibrium dialysis. The difference in c.p.m. between the two chambers was very reproducible between different sets of experiments. The variation between different values was never more than 5%.

Evaluation of the binding assay. The concentration of bound ligand was calculated as follows. At equilibrium at 30 °C, the value L_f (final concentration of free ligand) is the same on both sides of the dialysis membrane. Hence

$$L_0 = L_f + L_t + L_b$$

where L_0 is the initial concentration of ligand and L_b is the concentration of bound ligand.

Therefore

$$L_b = L_0 - 2L_f$$

Protein gel electrophoresis. Protein gel electrophoresis was performed as described by Laemmli (1970) and O'Farrel (1975). The separated proteins were visualized by the silver staining method of Blum *et al.* (1987). The native polyacrylamide gel was run in a similar manner without the addition of SDS and 2-mercaptoethanol.

Polyclonal antiserum. Polyclonal antiserum against the purified protein was raised in 5–7-month-old healthy rabbits. A bulk quantity of proline permease was purified from *C. albicans*. The aliquot from the sample was checked for homogeneity as well as for binding activity. The pure homogeneous protein sample (50–100 µg) suspended in PBS (0.145 M NaCl; 0.15 M sodium phosphate) was injected in the rabbits with the help of Freund's adjuvant. Two further boosters of immunogen (50 µg protein in PBS) were injected after every 10 d. The rabbits were bled a week after the last booster injection and the polyclonal antiserum was prepared from it. The specificity of the antiserum was determined by an ELISA and also by immunostaining of the electroblots (Catty & Raykundalia, 1988).

Protein estimation. Protein estimations were done using the Bradford (1976) method. Since the detergents used for solubilization of membrane proteins interfered with the Bradford assay, proteins were also estimated by the Lowry method and by measuring the A_{280} . The results of the protein estimation by different methods were compatible.

Reconstitution of purified proline permease into proteoliposomes. The proteoliposomes were reconstituted using a detergent-dialysis procedure as described by Höfer and co-workers (Mair & Höfer, 1988; Höfer *et al.*, 1991). Egg phosphatidylcholine (20 mg ml⁻¹) and 40 mg *n*-octyl β -D-glucopyranoside (molar phospholipid to detergent ratio = 0.5) were dissolved in 1 ml ethanol. The solution was evaporated to dryness in a vacuum evaporator by slowly rotating the evaporation flask. One millilitre of purified protein (1–2 µg) in reconstitution buffer containing 50 mM Tris/citrate (pH 4.5), 25 mM K₂SO₄ and 5 mM MgSO₄ was added to the phospholipid-detergent film and the flask was again rotated without applying vacuum until the solution became clear. The phospholipid-detergent solution was transferred into the 'Mini-Lipoprep' dialysis apparatus (Dianorm) and dialysed for 2 h (with a single change after 1 h) at 15 °C against 0.5 l reconstitution buffer using a dialysis membrane with a 1000 Da cut-off (no. 10.16; Dianorm). Reconstitution of proteoliposomes was completed after 2 h. To remove extravesicular K⁺ from the suspension of proteoliposomes, dialysis continued for a further 30 min against a fresh reconstitution buffer containing 25 mM Na₂SO₄ instead of K₂SO₄. This step significantly improved the energization of proteoliposomes upon the addition of valinomycin.

Measurement of L-[¹⁴C]proline uptake in the reconstituted proteoliposomes. The uptake of L-proline in reconstituted proteoliposomes was initiated by adding 100–150 µl proteoliposome suspension to the reaction mixture (total volume 1.0 ml) composed of 50 mM Tris/citrate (pH 4.5), 5 mM MgSO₄, 10 µM valinomycin (added after 60 s) and 10–100 µM L-[¹⁴C]proline at 30 °C. Aliquots of 100 µl of the reaction mixture at particular time periods were withdrawn and filtered through a nitrocellulose membrane filter (0.45 µm pore size; Sartorius). The filters were washed twice with 3 ml of the cold buffer (10 mM Tris/citrate, pH 4.5, and 5 mM MgSO₄), con-

taining the same concentration of unlabelled L-proline. The filters were then transferred into the scintillation cocktail and the amount of radioactivity retained in the proteoliposomes was measured using a Beckman beta liquid scintillation counter.

RESULTS

Functional reconstitution of symporters in yeast has so far been achieved mainly by using PM vesicles fused to proteoliposomes (Cooper, 1982; Höfer *et al.*, 1991; Serrano, 1988; Wach *et al.*, 1990). However, in this work, we have for the first time functionally reconstituted a purified proline permease protein of *C. albicans*.

Solubilization and purification of the proline permease

Purified PM was solubilized using Tween 40, which was able to solubilize more than 80% of the total membrane protein, and then loaded onto the L-proline-linked agarose matrix column equilibrated with washing buffer (0.1% Tween 40 in 100 mM Tris/citrate, pH 5.5). At this pH, proline permease showed maximum binding when checked with the equilibrium dialysis assay (Fig. 2d). The bound protein was eluted by the addition of 2 M NaCl containing 0.1% Tween 40 and each 5 ml fraction was analysed for L-proline binding. Out of five fractions, only the third fraction gave maximum binding with L-proline (Fig. 1). Various structural analogues of

Fig. 1. Elution profile of the L-proline affinity column. Solubilized proteins were fractionated on an L-proline agarose column as described in Methods. The arrow indicates the addition of 2 M NaCl for elution of bound protein from the column. Fractions of 5 ml were collected. The specific activity of L-proline binding (○) and protein concentration (●) were determined as described in Methods.

Table 1. Purification of the proline permease by affinity chromatography

Preparation	Protein (mg ml ⁻¹)	Total protein (mg)	Binding specific activity [nmol (mg protein) ⁻¹]*	Purification (-fold)†
CM	4.5	36.0	8.0	1.0
PM	3.5	10.5	10.5	1.3
Solubilized PM	1.0	7.0	15.0	1.9
Purified protein	0.002	0.01	860.0	107.5

* Proline binding was measured as described in Methods. The specific activity values represent the capacity of L-proline binding when the external concentration of L-proline was 10 μ M.

† Purification (-fold) was calculated by assuming the specific activity of binding of PM to be 1.3. The data are representative of a typical column run.

Fig. 2. SDS-PAGE of fractions at various steps of purification. (a) Lanes: 1, PM; 2, the purified protein fraction (PP) on a linear density gradient (6–20%). Molecular mass standards are marked. (b) Immunodetection of purified protein on a Western blot. Lanes: 1, PM; 2, the purified protein fraction (PP). (c) Native PAGE of the purified protein fraction on a linear gradient (6–20%) gel. The size of the PP on the native gel (67.0 kDa) is indicated by an arrow. (d) Specific binding of L-proline to the purified protein (●) and translocation of L-proline in reconstituted proteoliposomes (○) as a function of pH. The specific binding of L-proline and its uptake in proteoliposomes were performed as described in Methods.

L-proline, e.g. 2,4-dehydro-DL-proline (DHP) and azetidine-2-carboxylic acid (AZA), were also able to elute the bound protein but for convenience 2 M NaCl was routinely used for elution. There was more than 100-fold enhancement of specific binding of the purified protein. The yield of the purified permease represented 0.1% of the total PM protein (Table 1).

The purity of the final protein preparation was analysed by SDS-PAGE. The purified protein produced two diffuse bands of 64 and 67 kDa (Fig. 2a). By native PAGE, however, the purified protein produced only a

single band (Fig. 2c). The polyclonal antiserum raised against the purified permease also produced a single thick band on a Western blot, but it showed a dense zone between 63 and 67 kDa in the PM fraction (Fig. 2b). L-Proline binding to the purified protein has a sharp optimum at pH 5.5. There was practically no binding beyond this pH (Fig. 2d). The structural analogues of L-proline, i.e. DHP and AZA, were able to inhibit the binding competitively by more than 90% while D-proline had no significant effect. None of the other amino acids could significantly inhibit L-proline binding when applied at a 100-fold excess concentration (Table

Table 2. Substrate specificity of proline permease

Binding and reconstitution experiments were performed as described in Methods. L-[¹⁴C]Proline (10 μ M) and 100-fold concentrations of the competitors were used in these experiments. Amino acids other than those mentioned in the Table were also tried for the competition studies but none of them could inhibit its binding nor its uptake in the reconstituted proteoliposomes. Data are arithmetic mean $\pm$ SEM of three determinations.

Substrate	Proline uptake in <i>C. albicans</i> cells [nmol (mg dry wt) ⁻¹ s ⁻¹]	Inhibition of uptake (%)	Binding specific activity [nmol (mg purified protein) ⁻¹]	Inhibition of binding (%)	Proline uptake in reconstituted proteoliposomes [nmol (mg purified protein) ⁻¹ s ⁻¹]	Inhibition of uptake (%)
None	9.5 $\pm$ 2.0	—	842 $\pm$ 44.5	—	418 $\pm$ 13	—
DHP	1.0 $\pm$ 0.1	90.0	62 $\pm$ 22.0	93.0	70 $\pm$ 10	83.3
AZA	2.0 $\pm$ 0.2	79.0	85 $\pm$ 10.0	90.0	85 $\pm$ 4	80.0
L-Proline	1.2 $\pm$ 0.3	88.0	70 $\pm$ 20.0	92.0	62 $\pm$ 15	85.0
D-Proline	9.0 $\pm$ 3.0	5.0	785 $\pm$ 20.0	7.0	456 $\pm$ 50	—

2). These results are consistent with those of L-proline uptake in intact cells (Table 2) (Jayakumar *et al.*, 1978, 1979, 1981; Dabrowa & Howard, 1981; Holmes & Shepherd, 1987).

Half-saturation constant of L-proline binding to purified protein

The purified protein has an apparent K_m of 153 μ M for L-proline; the dissociation constant (K_d) calculated from a Scatchard plot was in the range 80–100 μ M (data not shown). In contrast to that of L-proline transport in intact cells, only monophasic binding could be detected for purified permease (Jayakumar *et al.*, 1978, 1979, 1981; Dabrowa & Howard, 1981; Holmes & Shepherd, 1987). *C. albicans* cells have two transport systems for L-proline; a high-affinity system with a K_m of 50–100 μ M and another one of low affinity with a K_m of about 1 mM.

Effect of N-ethylmaleimide (NEM) and cispentacin on proline binding

Various energy inhibitors (orthovanadate, azide and CCCP) or the addition of ATP did not inhibit or improve L-proline binding to purified permease, respectively (data not shown); however, NEM, a thiol group blocking reagent, inhibited the binding by 75% if added during the equilibrium dialysis along with the ligand. Fig. 3(a) shows that NEM did not replace bound proline when added after the binding was complete. Moreover, L-proline as well as its analogues protected the binding site from NEM inactivation.

Recently, cispentacin (2-aminocyclopentane-1-carboxylic acid), an antifungal isolated from *Bacillus cereus* and *Streptomyces setonii*, has been shown to have anticandidal activity. Cispentacin enters *C. albicans*

cells via both inducible proline permease and other amino acid permeases (Capobianco *et al.*, 1993). The entry of the drug is also energy-dependent and competitively inhibited by L-proline. Therefore, it was of interest to investigate drug interaction with the purified protein. Proline binding was inhibited by 75% in the presence of the drug. Similarly to the NEM effect, if cispentacin was added after binding of L-proline was complete, it had no effect on binding activity (Fig. 3a).

Reconstitution of proline permease in proteoliposomes

The purified permease demonstrated stereospecific binding and other characteristics which were consistent with earlier studies on uptake of L-proline in *C. albicans* cells (Dabrowa & Howard, 1981; Holmes & Shepherd, 1987; Jayakumar *et al.*, 1978, 1979, 1981). However, clear proof that it is purified proline permease lies with the fact that it can also mediate L-proline translocation in a reconstituted system. Therefore, the purified protein was reconstituted in proteoliposomes by the detergent-dialysis method described by Höfer *et al.* (1991). We have used phosphatidylethanolamine, phosphatidylserine and total *Candida* membrane lipids for L-proline binding and tried different lipid to protein ratios. In all cases, a 3:1 ratio gave maximum binding. Among all the lipids, phosphatidylcholine gave maximum (2.5-fold) enhancement of binding. The binding activation with other lipids was between 1.5 and 2.0-fold. Therefore, for reconstitution, only phosphatidylcholine was used. The enrichment of liposomes with purified protein could not be confirmed by SDS-PAGE. Due to the hydrophobic nature of the proteoliposomes, purified protein did not move on the gel. However, based on the specificity and kinetics of uptake of L-proline into proteoliposomes (discussed below), it was evident that the permease protein was incorporated into the liposomes in a right-side-out orientation.

Fig. 3. Effect of NEM and cispentacin on L-proline binding and its translocation. (a) Specific binding of L-proline to the purified protein in the presence of NEM (600 μM) and cispentacin (1 mM). 1, Control; 2, in the presence of NEM; 3, NEM addition after L-proline binding to the purified protein (10 min); 4, in the presence of cispentacin; 5, cispentacin addition after L-proline binding to the purified protein (10 min); 6, L-proline addition after cispentacin binding to the purified protein. (b) Effect of NEM and cispentacin on L-proline uptake in the reconstituted purified protein in proteoliposomes. 1, Control; 2, in the presence of NEM; 3, in the presence of cispentacin. L-[14C]Proline (92.5 MBq mmol⁻¹) (10 μM) was used for these experiments.

Fig. 4. L-Proline uptake in proteoliposomes reconstituted with the purified protein. (a) Uptake was initiated by the addition of 10 μM valinomycin after 60 s as indicated by the arrow (●); ○, uptake of L-proline in the absence of K⁺ salt; ■, uptake of L-proline in the presence of 25 μM CCCP. (b) L-Proline uptake in the reconstituted proteoliposomes with 10 μM valinomycin added from the start of the reaction (●) and L-proline uptake in the liposomes without reconstituting it with the purified protein (○).

Fig. 4(a) depicts a plot of a typical reconstitution experiment. The initial rate and maximum level of accumulation of proline were significantly increased

when valinomycin was added after 60 s incubation. Valinomycin specifically increases membrane permeability for K⁺; consequently, due to the existing K⁺ gradient (25 mM K⁺ inside, none added to the outside), a significant membrane potential ($\Delta\psi$) is generated across the vesicular membrane (inside negative) which, in turn, drives the accumulation of L-proline (Hirata *et al.*, 1971). Virtually no uptake of L-proline was observed when proteoliposomes were reconstituted without K⁺ or when $\Delta\psi$ generated by the K⁺ gradient was dissipated by an uncoupler, CCCP (Fig. 4a). When valinomycin was present in the uptake experiment from the beginning, the accumulation was immediate and it persisted over a period of several minutes. In the absence of the purified permease protein, no uptake into liposomes was seen, which again confirms the functionality of the reconstituted protein in proteoliposomes (Fig. 4b). The uptake of proline in reconstituted vesicles was maximal at pH 4.5 (Fig. 2d). The uptake could not be measured below pH 4.5 since proteoliposomes tend to collapse beyond this pH. It is pertinent to mention here that the uptake of L-proline in intact cells of *C. albicans* also showed an optimum pH between 4.5 and 5.5 (data not shown).

Kinetics of L-proline uptake in proteoliposomes

The kinetic parameters of the $\Delta\psi$ -driven L-proline transport were assessed by measuring the initial velocities of uptake in the L-proline concentration range between 5 and 100 μM. The half-saturation constant (K_m) of L-proline transport in the reconstituted system was 40 μM, which was in good agreement with that of the high-affinity component of proline transport (K_m 43 μM) obtained from studies with intact cells (data not shown). To ascertain the specificity of proline permease in proteoliposomes, uptake was measured in the presence of excess concentrations (100-fold) of either unlabelled D-proline or L-proline analogues DHP and AZA. D-Proline was without any effect on L-proline accumulation whereas the other two analogues competitively inhibited L-proline uptake (Table 2). Similar results were obtained with the intact cells of *C. albicans* where L-proline uptake was competitively inhibited by

DHP and AZA but remained unaffected by D-proline. Thus, the specificity of purified protein in reconstituted proteoliposomes was similar to that observed with intact cells (Table 2).

Effect of NEM and cispentacin on L-proline uptake in reconstituted proteoliposomes

Consistent with the binding results, NEM was also able to block the uptake of L-proline in reconstituted proteoliposomes (Fig. 3b). There was virtually no uptake of L-proline if NEM was added to proteoliposomes before energization was induced by the addition of valinomycin. Similarly, cispentacin also inhibited the uptake of L-proline in reconstituted proteoliposomes (Fig. 3b).

DISCUSSION

Despite the importance of nutrient transport in yeast, none of its transport proteins have been isolated and purified. This is largely due to the low content of the transport protein in the PM and partly because of the lack of a rapid assay to monitor purification. The present study describes for the first time the purification of the L-proline permease from *C. albicans* to homogeneity. The purified proline permease produced a single band of 67 kDa by both native PAGE and Western blotting. The resolution by SDS-PAGE, however, showed two bands (67 and 64 kDa) of diffuse appearance. Since the molecular mass of the purified permease protein on a native gel was 67 kDa, the possibility of two subunits being resolved by SDS-PAGE was excluded. Recently, a maltose transport protein was identified in *S. cerevisiae* which also gave two bands on SDS-PAGE (Van den Broek *et al.*, 1994). Although the proteins of the two bands differed in size, their sequence analysis revealed them to be homologous and highly hydrophobic. The antibodies against the Hxt2 glucose transporter also gave two bands on SDS-PAGE even after the blockage of glycosylation by tunicamycin (Wendell & Bisson, 1993). It appears from our results, as well as those of others, that the heterogeneity in size could be common among hydrophobic transport proteins of yeasts due to their interaction with SDS (Bernardo *et al.*, 1994; Isambert *et al.*, 1992). In this regard, it must be mentioned that no difference between the two bands of proline permease on SDS-PAGE was detected when stained for glycosylation (data not shown), thus excluding the heterogeneous glycosylation of the two bands as the reason for the appearance of the doublet.

The molecular mass of the purified protein was similar to that deduced from the nucleotide sequence of *PUT4* (proline permease) of *S. cerevisiae* (Vandenbol *et al.*, 1989). The size of the purified protein (67 kDa) obtained on a gradient gel, its binding specificity as well as its translocation properties in reconstituted proteoliposomes, are in agreement that the purified protein is a *C. albicans* proline permease.

Similarly to the *C. albicans* permease, the proline transporter of *Escherichia coli* in reconstituted vesicles has also been shown to be sensitive to NEM (Hanada *et al.*, 1992). NEM is able to inhibit proline binding and its subsequent translocation but its effect could be prevented if L-proline binding precedes NEM addition. Thus a cysteine residue(s) close to the binding site is probably essential for the proline permease activity of *C. albicans*.

The L-proline accumulation in the reconstituted proteoliposomes reported in this paper was energized by the membrane potential generated by the valinomycin-induced flux of K^+ out of the proteoliposomes. However, we were unable to energize L-proline accumulation by an applied pH gradient. This was very likely due to the nonspecific membrane permeability for protons leading to H^+ -equilibration before L-proline accumulation could be started (Mair & Höfer, 1988; Höfer *et al.*, 1991).

Cispentacin inhibits homoserine dehydrogenase and interferes with the synthesis of amino acids of the aspartate family. Since this enzyme is absent in animals, the drug can selectively kill prototrophic fungi (Yamaki *et al.*, 1990; Georgopapadokou & Walsh, 1994). The interaction of cispentacin with purified proline permease protein confirms that the drug competitively inhibits the binding and translocation of L-proline. Thus, knowledge of the transport mechanism of the drug and of its interaction with the purified protein would help in designing better synthetic drugs.

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